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Award Number: DAMD17-98-1-8507

TITLE: New Strategy for the Redirection of Cytolytic T Lymphocytes to Prostate Tumors

PRINCIPAL INVESTIGATOR: Zelig Eshhar, Ph.D.

CONTRACTING ORGANIZATION: The Weizmann Institute of Science
                            76100 Rehovot, Israel

REPORT DATE: February 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                          Distribution Unlimited

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20010723 065

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2001	Final (1 Aug 98 - 31 Jan 01)

4. TITLE AND SUBTITLE
New Strategy for the Redirection of Cytolytic T Lymphocytes to Prostate Tumors

5. FUNDING-NUMBERS
DAMD17-98-1-8507

6. AUTHOR(S)
Zelig Eshhar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Weizmann Institute of Science
76100 Rehovot, Israel

E-Mail: zelig.eshhar@weizmann.ac.il

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We developed an immunotherapy system using chimeric T cell receptors to redirect T cells to tumors. The objective of this research project has been to study the ability of effector lymphocytes expressing chimeric receptors (CR) to eliminate prostate cancer (PC). Previously we showed that such T cells can kill target PC cells in vitro. Now we report on successful attempts to reject PC xenografts in vivo in SCID mice. Retrovectors harboring HER2-specific CR genes where the scFv is linked to CD28 and the FcRγ are used to efficiently transduce activated human lymphocytes. When injected into the CWR22 PC xenograft, about 30% of mice treated with the anti-HER2 (and not control anti-TNP), a complete tumor rejection was observed, evident by disappearance of tumor concurrently with a decrease in PSA levels. In several of the treated mice where the xenograft growth was resumed, we found that the re-growing tumor cells lost their ability to bind the particular anti-HER2 antibody that served to construct the CR. These cells could still bind to an antibody directed to another HER2 epitope. IL-2 was found to be required for more efficient cancer rejection. This therapeutic strategy may allow new approach towards the adoptive immunotherapy of localized PC.

14. SUBJECT TERMS
T-body approach, immunotherapy, chimeric receptors, prostate cancer xenografts, prostate specific antigens

15. NUMBER OF PAGES
80

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The immunotherapy of cancer and viral diseases has several advantages over the classical therapies of chemotherapy and radiation. Immunotherapy is expected to be more specific for diseased tissue, and less toxic to the healthy organs of the patient. Nonetheless, in most cancer cases the immune system of the patient fails to cure the disease. To overcome this problem and to enhance the ability of the patient's own immune cells to fight fatal diseases, our group has pioneered the 'T-body' approach. In this approach, patient-derived effector cells (T or natural killer (NK) cells) are transduced with gene encoding a chimeric receptor comprised of a ligand or antibody-derived variable regions specific for a tumor antigen and linked to an effector cell activation molecule, such as the Fc receptor gamma chain. The transduced effector cells are thereby redirected to tumor cells, and upon reintroduction to the patient, are expected to kill target cells expressing the selected antigen. This approach combines the ability of T cells to home to, penetrate, and eliminate large solid tumors with the ability of antibodies to recognize a pre-selected antigen with high specificity and affinity without MHC restriction. The overall objective of the project is to refine and optimize the T-body approach and further develop it towards cell mediated immunotherapy of prostate cancer. Specifically, we are optimizing the composition of the chimeric receptor, directing it towards prostate cancer antigen by the generation of new scFv against prostate cancer surface antigens and improve the means of introducing such chimeric receptors into human T cells.

Body

Prostate cancer is the most common diagnosed malignancy and the second leading cause of cancer death in American men today. Metastatic prostate cancer has a 5 year survival rate of about 30%. Radical prostatectomy and radiation therapies are currently the principal curative treatment modalities for organ confined disease. External beam radiation therapy and radical prostatectomy used alone have significant limitations in eradicating locally advanced tumor (1). The treatment of choice for advanced disease is hormonal therapy, which is effective for androgen-sensitive tumors. However, treatment options for hormone-resistant disease are, at present, limited. Moreover, local and distant failure after radiotherapy or radical prostatectomy as monotherapies are not uncommon, since many of the clinically defined localized tumors harbor more advanced pathological disease. Hence there is a role for neoadjuvant treatments to improve the probability of local cancer control.

The resistance of advanced prostate cancer to conventional therapy, combined with the presence of defined tumor- or tissue-associated antigens on prostate tumors, makes this malignancy an ideal candidate for an immunotherapeutic approach to treatment. In addition, prostate cancer displays several unique features that underlie the potentiality of tumor immunotherapy in achieving this therapeutic goal:

a) Although prostate cancer is a visceral tumor, the primary tumor site within the prostate is anatomically easy to access and image. Therefore, various effectors including immune cells can be readily and accurately transferred directly into the tumor, assisted by routine transrectal ultrasonography (TRUS) of the prostate.

b) Prostate cancer has a unique tumor marker: Prostate Specific Antigen (PSA) and its serum levels correlates with the disease stage (2). PSA serves not only for screening and detection of prostate cancer, but also to monitor prostate cancer progression. PSA therefore can contribute significantly in evaluating the immune response against the tumor.

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c) Non-specific immuno-destruction of the prostate is beneficial: Immunotherapy is not absolutely specific due to deficiency of tumor specific antigens. Thus most of the prostate tumor associated antigens are also expressed in normal prostate tissue. Since the prostate is not a vital organ and can therefore be removed in its entirety, collateral immune damage to non-cancerous prostatic tissue ("biological prostatectomy") is not critical and may even help to reduce local cancer recurrence.

The cellular arm of the immune system is the principal defense mechanism by which the body rejects foreign, infected or transformed tissues. Although cytotoxic lymphocytes have been used successfully to treat tumor patients, their use for adoptive immunotherapy either suffers from a lack of specificity, as in the case of lymphokine-activated killer cells, or is limited to only a few malignancies (e.g. melanoma and renal cell carcinoma) from which tumor-infiltrating lymphocytes can be derived. On the other hand, antibodies can be readily raised against various antigens associated with cancerous tissue. However, the inefficient accessibility of antibodies to solid tumors limits their therapeutical use. A new approach which we have recently developed combines the specificity of antibodies with the efficient effector-cell properties of T cells. It takes advantage of the similarity in the genomic organization and molecular structure of the TCR and Ig molecules and utilizes pairs of chimeric genes , each composed of one of the variable regions (VH or VL) of a specific antibody linked to a constant domain (Cα or Cβ) of the TCR chains. Although such chimeric receptor genes could be functionally expressed in T cell hybridomas and tumors, it requires co-expression of two transgenes, a practically very inefficient endeavour for normal T cells. To overcome this difficulty, and extend this approach (which we nicknamed "T-body") to include additional receptor molecules and cells, we have adopted the single chain Fv (scFv) design that joins the antibody's VH and VL via a synthetic linker to yield a functional Fv.which was then linked with the γ or ζ subunits (of the Fc and CD3 receptor complexes, respectively)(scFvRγ/ζ). The resulting polypeptide is a functional receptor which was properly expressed on the cell surface as a

homodimer, bound hapten, and triggered T cell activation. The "T-body" approach combines the effector immune functions of the T cells with the ability of antibodies or ligands to recognize a pre-selected antigen with high specificity and without MHC restriction. The latter is of a special importance, since prostate cancer cells often down-regulate the expression of MHC molecules (3, 4). In-vitro experiments from our laboratory (5) and elsewhere (6, 7) have demonstrated the efficiency of this approach. The differential expression of growth factor receptors on cancer cells as compared to normal tissues, their cell surface localization and their active role in the malignant process make these proteins a suitable target for tumor immunotherapy. Overexpression of ErbB-2 (HER-2) has been detected in up to 82% of prostate cancers (8). More importantly, HER-2 expression has been shown to increase as androgen dependence decreases both in vitro (9) and in vivo (10).

Our research went along two parallel lines: First involved in the generation of prostate specific monoclonal antibodies, cloning of scFv of selected antibodies, construction of chimeric receptor genes and their functional expression in cytotoxic murine T cell hybridoma. In the second line, we aimed at the development of vectors and procedures for functional expression of cloned chimeric receptor genes into human effector lymphocytes. At a very early stage of the research, using several human prostate cancer lines and a few xenografts, we found that the p25 series of antibodies we have received from the ATCC failed to stain surface antigens with most of the samples. In addition, we found out that the anti- extracellular region of PSMA antibodies could not be available to us. We have therefore diverted our attempts to the production of new sets of monoclonal antibodies towards known (PSMA and HER2) as well as new prostate specific antigens (PSCA and STEAP). In doing these we preferred the faithful hybridoma technology on the largely experimental phage display technology.

Cloning and Expression of PSMA

PSMA was found as the antigen for the antibody 7E11 (11). It is a type II membrane protein so its carboxyl terminus is extracellular with a transmembrane domain and a short intracellular domain. It is made up of 750 amino acids giving it a molecular weight of 84 kDa before glycosylation. There are 9 potential glycosylation sites. In mammalian cells it has a molecular weight of about 100 kDa. It has an enzymatic activity of a glutamate carboxypeptidase (12) (Figure 1). It has folate hydrolase activity and has been given the gene name FOLH1. It also has N-acetylated alpha-linked acidic dipeptidase (NAALADase) activity whereby it catalyzes the hydrolysis of N-acetyl-L-aspartyl-L-glutamate (NAAG), a neuropeptide, to release glutamate, a neurotransmitter implicated in a number of excitotoxic neurodegenerative disorders (13). Two other proteins form a closely related gene family together with PSMA. Human ileal peptidase is NAALADase- "like" (NAALADase L) since it is a peptidase but is unable to hydrolyze NAAG. NAALADase II is the third member of this family (14). This family of genes has homology to the transferrin receptor genes. Since the crystal structure of the transferrin receptor is known, it can be used to give a general picture of the structure of PSMA (15). It had been observed that PSMA had a domain with homology to aminopeptidases. The unexpected result of the crystal structure is that the peptidase domain is split into two pieces by a PA or apical domain (16). PSMA has also been found in the neovasculature where it may serve as a target for anti-angiogenesis (17).

RNA was isolated from the LNCaP human prostate cancer cell line using the Ultraspec™ RNA isolation system (Biotecx Laboratories, Houston, TX). Reverse transcription was done using a Reverse Transcription System (Promega, Madison, Wisconsin) with 0.5 µg of oligo(dT) as primer for a 20 µl reaction mixture. The resulting cDNA was used as template to PCR the coding sequence of PSMA in two pieces and PSCA. The primers have restriction sites to clone the PCR product into pcDNA3 (Table 1).

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Specifically the nucleotide sequence used was from Genbank (gb:humpsm). The protein sequence is in Swissprot (sw:psm_human). The 5' PCR primer (#26641) started 8 nucleotides before the initiating ATG. Before that sequence a Hind III site (relevant restriction sites are underlined) was added to facilitate cloning. The nucleotide sequence representing the amino terminal fragment of PSMA was isolated as a PCR fragment down to the pre-existing EcoR I site (nucleotides 1572-1577) in the PSMA sequence (amino acids 437-439). When cloned into pcDNA3 this gave plasmid #342. The carboxyl terminal fragment was cloned by using for PCR the primer #26643 from the pre-existing EcoR I site mentioned above and primer #26644 to the carboxyl terminus of the protein (amino acid 750) where a BamH I site was added to facilitate cloning. When cloned into pcDNA3 this gave plasmid #344.

Each fragment was excised and cloned into pcDNA3-HA to give plasmid #350 with the entire PSMA molecule fused to an epitope tag from influenza hemagglutinin (HA-tag). This plasmid has the PSMA-HA coding sequence with a CMV promoter and a bovine growth hormone poly(A) site (Figure 2). It has the bacterial neo gene for selection by G418 in mammalian cells and an amp gene for selection with ampicillin in bacteria. This plasmid was transfected into mouse myeloma NS0 cells but no G418 resistant transfectants were obtained.

The PSMA coding sequence was also cloned into the pEGFP-C1 plasmid (Clontech) to fuse GFP to the amino terminus of PSMA (Figure 3). Since PSMA is a Type II membrane protein, this would orient GFP into the cytoplasm and give a PSMA which does not need an antibody to be detected. The vector was prepared by cutting the pEGFP-C1 plasmid with EcoR I, blunting the EcoR I site with Klenow fragment of E. coli DNA polymerase by filling in with nucleotides, and cutting with BamH I. The vector was purified on an agarose gel and ligated to the PSMA sequence digested with Msc I which cuts in the 13th codon deleting most of the cytoplasmic region and BamH I at the end of the PSMA coding sequence. Digestion of the final plasmid with BamH I and EcoR I gave the expected fragment sizes, 4700 bp, 1278 bp, and 939 bp. This plasmid was assigned the designation

#514. It carries the neo gene to give G-418 resistance in mammalian cells. It was transfected into Chinese Hamster Ovary (CHO) cells and mouse myeloma NS0 cells.

For expression in *E. coli* fragments of the PSMA coding sequence were cloned into pGEX-3X to produce fusions to glutathione-S-transferase (GST). These fusions are expressed from an inducible hybrid trp-lac promoter. The plasmid contains the lacI gene to repress the lac promoter until induction by IPTG. The PSMA clone was used as a template in a PCR reaction using primers #27909 and #26644 to give a fragment coding from amino acid 274 to amino acid 750 (477 aa) which is in plasmid #371 (Figure 1). This protein fragment is from the amino terminus of the carboxypeptidase domain, which is now fused to the 218 amino terminal amino acids of glutathione-S-transferase to give a protein about 42 kDa (Figure 4).

Since PSMA is Type II membrane protein, a fragment from the carboxyl terminal end was also expressed in pGEX. Primer #29076 gave a PCR fragment, which coded for a protein segment starting from amino acid 599, just after the last cysteine so that the protein fragment would not have a cysteine in it. Primer #26644 coded to last amino acid, number 750. When cloned into pGEX it produced plasmid #380 (Figure 1). This fragment is 152 amino acids and should give a fusion protein of about 40 kDa.

When transformed into bacteria TG-1 and induction by IPTG both plasmids produced proteins of the appropriate sizes in insoluble inclusion bodies.

Two plasmids were made for bacterial expression using the strong T7 promoter in the pET28 vector. It contains a His tag to enable purification of inclusion body proteins under denaturing conditions. Similarly to the pGEX vectors, one expression construct was made with the 477 amino acid carboxyl terminal fragment and one with a shorter 164 amino acid carboxyl terminal fragment.

Upon the determination of the structure of the transferrin receptor, it became clear that the carboxyl terminal fragment we were expressing was close to the membrane and not protruding. In fact part of it was involved with dimerization of the transferrin receptor and would not be available

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to an antibody (or T cell) coming from the outside. As a result of this analysis, we attempted the expression of the protease-associated or apical domain which extends from the membrane. Four different bacterial expression systems were tested. The first is the GST fusion system (18). As with the helical domain, the PA domain were well expressed but insoluble. The GST-apical domain could be solubilized in 8M urea and the urea removed by stepwise dilution and dialysis with the maintenance of solubility.

In order to produce soluble protein these domains were fused to the nusA gene of E. coli. This protein solubilizes proteins to which it is attached (19). This concept is utilized in the Novagen vector pet43. An additional advantage of this vector is that the gene is driven by the strong T7 RNA polymerase promoter. This did not solubilize the helical domain, although it was highly expressed in the insoluble fraction. A third vector tried was the Clontech pHAT-GFPuv (20) which contains a his tag for purification and a GFP for visualization (21). The insoluble helical domain caused loss of fluorescence. A fourth expression vector from New England Biolabs uses the strong T7 promoter, a chitin binding domain for purification and an intein for removal of the expressed protein from the intein containing the chitin binding domain. Again the helical domain was insoluble, but could be solubilized in 8M urea and the urea removed by stepwise dilution and dialysis with the maintenance of solubility (Figure 5).

Additional fragments were used to attempt expression as a fusion to human Ig as was succeessful with PSCA (see below), however expression was not obtained.

Cloning and Expression of PSCA

Prostate stem cell antigen (PSCA) is a GPI-linked, Ly6-like protein (22). The mature protein has only 79 amino acids but with 4 N-glycosylation sites, it has a molecular weight of 24 kDa. It has 4 disulfide bonds like CD59 and cobra toxins. The sequence used to clone it came from Genbank (gb:af043498). PSCA was cloned using primers #26586 and #26587. The 5' primer, #26586,

included a Hind III site and 11 nucleotides before the initiating ATG codon. The 3' primer, #26587, placed a Xho I site after the termination codon. When this PCR fragment was cloned into similarly cut BamH I-Xho I pcDNA3 it resulted in plasmid #340. Our clone has a T at position 329 instead of a C. This is a silent change as in both cases it codes for alanine in the GPI signal. This was cloned into pcDNA3 (Figure 6).

For expression of the unmodified protein without the GPI linkage but rather as a transmembrane protein in mammalian cells this plasmid was used as the template in a PCR reaction using primers #27190 and # 27191. Primer #27190 placed a Xba I site before the amino acid sequence LQPG that is the amino terminus of the mature protein. Primer #27191 placed a Bst EII site after the NASGAHA sequence near the carboxyl terminus of the PSCA protein thereby removing the hydrophobic domain signaling GPI addition. This fragment was cloned into a RSV-gamma expression vector to give plasmid #353. In this vector, the mature PSCA protein has a mouse immunoglobulin light chain leader and a human IgεR gamma chain transmembrane region. Between the PSCA and the membrane is a YOL epitope tag and an IgG hinge spacer (Figure 7).

A vector to express PSCA with a YOL epitope tag was produced. The carboxyl terminus of the tag was placed on with primer #27237. The amino terminus of the tag was placed on with primer #27186. The PSCA with the YOL tag was cloned into the pcDNA3 vector (Figure 8).

PSCA was expressed as a fusion to human IgG constant region sequences to produce a PSCA-IgG fusion protein. For expression as a PSCA-IgG chimeric protein, primers #26586 at the extreme amino terminus of the protein and # 27456 were used. Primer #27456 places a Bam HI site before the hydrophobic GPI signal at the carboxyl end of the PSCA protein. This enables the fusion to the IgG constant region in the expression vector driven by the CMV promoter (Figure 9). This plasmid (#360) was used to transfect by means of calcium phosphate 293T cells, human embryonic kidney cells that have been transformed with adenovirus and express SV40 T antigen. After 7 hours

the medium was collected. This protein was purified on a Protein A column and used to immunize mice.

For bacterial expression of PSCA, its coding sequence was cloned into pGEX-3X using primers 28254 and 27748. This places a Bam HI restriction site in front of six amino acids before the beginning of the mature PSCA sequence and a EcoR I site at the end of the coding sequence for the mature PSCA protein enabling insertion into the pGEX-3X vector and production of a GST-PSCA fusion protein. These 85 amino acids fused to the GST portion gives a protein of a molecular weight of 33 kDa. Upon induction with IPTG the fusion protein is expressed and found in the insoluble inclusion body fraction.

Another antigen is the six-transmembrane epithelial antigen of the prostate or STEAP which is a 339-amino acid protein with 6 potential membrane-spanning regions flanked by hydrophilic N- and C-terminal domains (23). This structure suggests a potential function as a channel or transporter protein and indeed it is located on the surface supporting STEAP as a cell surface tumor-antigen target for prostate cancer therapy. We have cloned STEAP from a WISH-2 prostate tumor xenograft developed in this lab (24). It was inserted into CMV IE promoter-based plasmids with either the HA epitope tag plasmid (plasmid #445) (Figure 10) or with RFP (plasmid #449) on the carboxyl terminus.

Immunization of mice for monoclonal antibody production

Mice were immunized with bacterially expressed GST-PSMA fusions and PSCA-Ig using standard protocols. To evaluate the specificity and titer of antibodies produced following such immunizations we employed two assays: immunofluorescence staining of viable cells, and ELISA, using glutaraldehyde-fixed cells. FACS was used to measure the ability of antibodies in the mouse sera to stain LNCaP cells. Serum from mouse PSCA#5 stained LNCaP nicely at a dilution of 1:450

whereas normal mouse serum only stained at a dilution of 1:50. SKBr3 cells, a human mammary epithelial tumor cell line which do not express PSCA or PSMA, do not stain.

Serum from mouse PSMAII3 stained LNCaP nicely at a dilution of 1:1350, whereas it stained SKBr3 at a dilution of 1:50.

These sera were tested in a whole cell ELISA. An ELISA plate was coated with a solution of 2 mg/ml of poly-lysine. Then LNCaP cells were allowed to bind. After washing with PBS glutaraldehyde (0.25%) was added for 15 minutes at 37°C. After rinsing with PBS the plates were blocked with 1% BSA. Dilutions of serum were added for 1 hour at 37°C, the plates were washed and developed with peroxidase-conjugated goat anti-mouse antibody. Both sera reacted with LNCaP as the PSCA serum and the PSMA serum reacted more than normal mouse serum (Figure 11).

These mice serve now as the source of immune cells to generate hybridomas and monoclonal antibodies.

Retroviral Constructs

Moloney based

The BULLET system of Bolhuis and colleagues was attempted first (25). This system involves the transient transfection of three plasmids to make a transducing retrovirus. One plasmid codes for the envelope protein, which could be from an ecotropic virus, an amphotropic virus, or from the gibbon ape leukemia virus (GaLV). Another plasmid supplies the gag and pol proteins. The transfer vector with the LTRs and packaging signal carries an insert with the gene of interest (Figure 12). In our experiments this insert was specially designed. The scFv used in our initial experiments was from an antibody with specificity to trinitrophenol (TNP). This facilitates the testing of the genetically modified lymphocytes to kill a wide variety of cells as any cell can be made a target simply by treating with picryl chloride to label the cell surface with the trinitrophenyl (TNP) group. Another advantage of this scFv is that we have available an anti-idiotypic monoclonal antibody for

detecting by FACS expression of this scFv on the cell surface. Genetically fused to this scFv is the co-stimulatory molecule CD28 to enable the production of a chimeric protein. This provides spacing of the scFv from the cell surface and permits the formation of heterodimers with endogenous CD28 molecules. The CD28 molecule has previously been used in chimeric receptor constructs (26). In addition, CD28 signaling has been shown to prevent apoptosis of lymphocytes. Attached to the carboxyl terminus is the intracellular portion of the human IgE receptor FcεRI γ chain. This contains the signaling portion of the receptor and completes the gene for the chimeric T cell receptor. In order to enable better tracking of retroviral expression the gene for green fluorescent protein (GFP) was placed downstream of the chimeric receptor gene separated by an internal ribosomal entry site (IRES) to permit expression of the GFP from the same transcript as the chimeric receptor. Retroviruses with a single transcript containing a desired gene and GFP driven by an IRES have been made previously (27). In our case the GFP gene was placed in the retroviral vector pSAM-EN (28) in place of the drug resistance gene and the chimeric receptor gene was inserted into the cloning site before the IRES. Lack of a second promoter avoids the problem of promoter interference. It also enables linked expression so that measuring the fluorescence of the GFP can assess expression of the scFv. Transduced cells can be observed visually through a fluorescent microscope. This will permit the tracking of transduced cells in vivo (29).

Better results were obtained with a stable packaging system than with the transient packaging system. A variety of different stable packaging cells were tried. The FLY series is based on the human cell line HT-1060 (30). Packaging cell lines of these cells are available producing RD114, amphotropic, or GaLV envelope. Murine based packaging cell lines are available based on the 3T3 cell line. These cell lines are available to produce ecotropic, amphotropic or GaLV envelope proteins. These packaging cell lines were infected with virus from the Ping-Pong transfection (31)(Figure 10). PG13 (32) providing the GaLV in a 3T3 based packaging cell line gave the highest expression of GFP. PA317 providing the amphotropic envelope worked almost as well (Table II).

The packaging cells can be sorted on the basis of the green fluorescence to give a population of cells producing virus. They can also be cloned by sorting cells into individual wells to give a virus producing line.

For optimal transduction of human peripheral blood lymphocytes with retroviruses the lymphocytes are activated. They are grown in RPMI with fetal calf serum (FCS) without IL-2 with plastic bound anti-CD3 and anti-CD28 antibodies for 48 hours. They are then infected with supernatant containing virus from the packaging cell line on plates coated with recombinant fibronectin fragments (Retrofectin™) (33). The infection is done in the presence of 50 U/ml of IL-2 for 5 hours. The cells are then grown for 24 hours in RPMI-FCS with 100 U/ml of IL-2 and the infection repeated for another 5 hours (Table 2). Low concentrations of IL-2 were used to prevent to propagation of natural killer cells. Higher concentrations are used for the production of lymphokine-activated killer (LAK) cells (34). LNCaP cells are deficient in their expression of MHC which would interfere with its ability to inhibit NK cell activity (3).

The lymphocytes can be shown to express GFP by the green fluorescence and the scFv-CD28-Fcεγ receptor by staining the cells with a monoclonal antibody specific for the scFv. The stainings correlated in intensity verifying that determination of GFP expression is a suitable surrogate for the measurement of chimeric receptor expression (Figure 13).

Measuring the production of IL-2 in response to stimulation tested the functionality of the transduced receptor. Infected lymphocytes were starved for IL-2 and stimulated with plastic-bound TNP-fowl IgG. The lymphocytes responded to stimulation by producing IL-2 enabling lymphocyte survival. This survival was measured by an MTT assay (35). Different loadings of TNP were used (Figure 14).

These lymphocytes were able to kill tumor cell lines including LNCaP after they were TNP-ylated. The first cell line tried was the Burkitt tumor-derived, human B lymphoblastoid cell line, Raji which is resistant to NK killing although sensitive to killing by lymphokine-activated killer (LAK)

cells. Efficient killing was obtained in only 4 hours at an effector to target ratios as low as 2:1 (Figure 15). Other B lymphoblastoid cell lines, such as the human Daudi and the Balb/C mouse A20, were also good targets for lymphocyte killing as was the prostate adenocarcinoma epithelial LNCaP (36) (Figure 16).

Based on the success with the anti-TNP scFv two other targeting molecules were placed into the same transfer vector in place of the scFv. These targeting molecules were directed towards the heregulin receptor, which is composed of HER2 and HER3 or HER4. HER2/neu, which was shown to be expressed on cultured human prostate cancer cell lines including LNCaP, DU-145, and PC-3 One is a scFv targeting HER2 based on a monoclonal antibody (5). Out of a panel of monoclonal antibodies (mAbs) specific to the extracellular portion of the Neu/HER2 protein (37) we selected mAb N29 which significantly inhibited the tumorigenic growth of neu/HER2 transfected fibroblasts in nude mice, and induced phenotypic differentiation of various cultured breast cell lines (38). Heregulin itself has been used as the targeting molecule for toxins, viruses and cells (6, 39, 40). In our case we have a YOL epitope tag to facilitate the detection of chimeric receptor expression by a monoclonal antibody (41). Indeed, human PBL, activated with anti- CD28+CD3 antibodies and transduced by supernatants of retrovector producing packaging cells, expressed the chimeric receptors and GFP (35-70% of the cells were stained, data not shown). HER1, HER2, and HER3 are consistently found in cell lines and tumor samples, but HER4 is not (42). The LNCaP cell line contains relatively high levels of both HER2 and HER3 compared to other prostate tumors (40, 43). The NDF-directed and the N29-directed human lymphocytes exhibit higher killing of LNCaP than non-directed lymphocytes (Figures 15, 16). However high background killing is evident in these samples, especially at high effector to target ratios. This may be the result of NK killing of the LNCaP as it is deficient in MHC which blocks NK killing (3). Chinese hamster ovary (CHO) cells and 32D, a mouse hematopoietic cell line (44) display lower background killing. These cells have been transfected with HER2 together with HER3 (45, 46). When these cells are used as targets,

HER2/3 dependent killing is much clearer (Figures 16, 17). Sensitivity is correlated to the level of expression of HER2/3. The CHO cells with HER2 and HER3 contain 60,000 NDF receptors with an affinity of 2 nM (46). The 32D cells with HER2 and HER3 express about 12,000 NDF receptors (45). LNCaP has about 15% the amount of HER2 that SK-BR3 has or 18-fold over-expression (40, 47).

In vivo effects of transduced human lymphocytes in an animal model

As a representative target for human prostate cancer we elected to use the CWR22 human PC xenograft (a generous gift from Prof. T Pretlow). CWR22 is an androgen dependent human PC adenocarcinoma xenograft, over-expressing the HER2 growth factor receptor (42). The tumor secretes PSA to the tumor bearing mouse sera. Seven to 10 week old male (c.b-17/Icr-scid-bg) SCID mice (Harlan laboratories) were transplanted subcutaneously (s.c.) with 90-day slow released testosterone pellets (Innovative Research of America Sarasota FL). Equal burden of xenograft tissue or cells (depending on the specific experiments) were transplanted s.c. to the testosterone-supplemented mice. The tumor tissue or cells were co-transplanted with Matrigel basement membrane matrix (Becton Dickinson Bedford MA) as was previously described by Pretlow et al. (48). Tumor growth was followed by measuring tumor volume and serum PSA levels. Mice bearing established equal tumors by means of both tumor volume and PSA secretion were randomized to the different experimental groups. In some experiments in which continuous systemic administration of interleukin-2 (IL-2) was desired, mini-osmotic pumps (ALZA Corporation CA) were transplanted intra-peritoneal 24-72 hours before the first intra-tumoral injection. Each pump was loaded with recombinant human IL-2 (R&D System Minneapolis MN), planned to continuously release 113U/hour for a week (Exp.2) or two weeks (Exp.3&4).

Established tumors were evident by detectable serum PSA and measurable tumor (Table III). Once tumors were established, mice were randomized to an experimental group that received the

therapeutic chimeric receptor-bearing lymphocytes (anti-HER2), to a control group that received irrelevant chimeric receptor-bearing lymphocytes (anti-TNP) and to another control group that received direct injections of only HBSS which served as the suspension medium for the injected lymphocytes. The latter group represents the natural progression of the tumor. In experiment 2 that tested the role of simultaneous administration of IL-2 each of the above mentioned groups was divided into 2 separate subgroups: one that received and one that did not receive IL-2. The chimeric receptor-bearing lymphocytes were suspended in HBSS at the desired concentration (usually 10^7 lymphocytes in 150 ul). Lymphocytes (10^7) were directly injected into the tumor for 3 to 5 consecutive days. End points to evaluate treatment efficacy included: tumor volume (as determined by caliper measurements of tumor length, widths and height), serum PSA levels and animal survival as defined by both actuarial death and by the percentage of living mice with tumor weight of less than 10% of body weight. Above this point the mice were euthanized according to the ethical rules of the committee.

FACS analysis:

FACS analysis was done for the expression of the transgene. Since the chimeric construct transgene contains the gene for GFP, the expression of GFP was used to determine the transgene expression in the transfected lymphocytes. FACS analysis for GFP expression was done in several time points following the completion of the transfection. As control mock transfected lymphocytes from the same experiments were used. Table II summarizes the mean transgene expression in the different experiments.

FACS analysis for HER2 was done by washing single-cell suspensions made from the xenograft and incubating with primary antibody N29 or L96 for HER2 (obtained from Prof. Y. Yarden) (49) or an irrelevant, isotype-matched U76.6, anti-dinitrophenol monoclonal antibody. The samples were then washed and incubated with the secondary FITC labeled anti-mouse antibody.

Stained cells were resuspended in propidium iodide to identify and exclude dead cells. Analysis was performed on the FACSCAN flow cytometer (Becton Dickinson).

Enzyme-linked immunosorbent assay (ELISA) for PSA:

A sample of 200 ul of blood was drawn from the mice at different time intervals and the serum separated. Levels of serum PSA were measured using standard ELISA kits (Diagnostic Products Corp., CA).

Results:

Experiment 1

We started our in-vivo experiments by testing the effect of the "T-bodies" on a relatively large tumor burden as was also evident by high levels of PSA in the mouse sera (mean 8.6ng/ml). There were 3 experimental groups (each of 7 animals): 10^7 anti-HER2 (therapeutic) or anti-TNP (control) chimeric receptor-bearing lymphocytes, suspended in 150 υl HBSS, were injected directly into the tumors in 5 consecutive days (group 1 and 2 respectively). In order to further evaluate the effect of the treatment, a second control group of mice underwent simultaneous direct intratumoral injection of 150 μl HBSS alone. This group represents the natural progression of the tumor. In this experiment direct intratumoral injection of anti-HER2 chimeric receptor-bearing lymphocytes to a relatively large mass of localized PC resulted in delayed and reduced tumor growth (p=0.018, Figure 20) and PSA secretion (Figure 21). Treatment with anti-HER2 chimeric receptor-bearing lymphocytes resulted in an 50% animal survival of 10.5 weeks which was double the 50% survival in both control groups which was 5.5 weeks (p=0.01, Figure 22). The relatively short survival reflects the aggressive nature of this tumor and the large pre-treatment tumor burden. Follow-up on

tumor volume was performed up to 7 weeks post-treatment, since by that time the mean tumor volume in the control groups was already larger than 2000 mm^3 (10% of the mouse body weight).

Experiment 2

We next intended to evaluate the treatment efficacy on relatively early tumors with small tumor burden. In addition, along with our attempts to optimize the treatment protocol, the effect of simultaneous systemic administration of IL-2, that was already shown to enhance effector function of adoptively transferred immune cells in mouse models (50), was tested. Therefore, only mice with small tumors and low serum PSA levels were included.

By applying this protocol we could achieve cure in animals that were treated by the combination of direct intratumoral injection of anti-HER2 chimeric receptor-bearing lymphocytes in parallel to continuous systemic administration of IL-2.

FACS analysis of the expression of HER2 epitopes that are recognized by the N29 antibody's variable region demonstrated that tumors that were treated and initially responded to the therapeutic protocol, but eventually regrew, expressed smaller amount of N29 epitopes on their surface as compared to tumors in the control groups. This may indicate that failure to maintain the primary response may result from a selection and growth of tumor cells whose N29 epitope was not accessible to the redirected lymphocytes.

IL-2 by itself did not induce any therapeutic effect since animals treated with intra-tumoral injection of HBSS together with systemic administration of IL-2 showed the same pattern of tumor growth rate and PSA secretion as animals in the control groups that were treated with anti-TNP chimeric receptor-bearing lymphocytes plus systemic administration of IL-2, or anti-TNP chimeric receptor bearing lymphocytes alone (Figures 23 & 24). In this experiment we could not demonstrate any therapeutic advantage to the "T-bodies" without accompanied systemic administration of IL-2.

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Experiment 3

Experiment 3 was intended to substantiate the effectiveness of the combination of direct intratumoral injection of anti-HER2 "T-bodies" with systemic administration of IL-2 as in experiment 2 however now on more advanced localized tumors (as reflected by larger mean tumor mass of 140 mm^3 and mean serum PSA of 18.6 ng/ml).

A clear short initial reduction in tumor volume was evident, followed by a substantial tumor growth retardation as compared to both control groups (p=0.03, Figure 6). This effect was achieved on quite advanced localized tumors as both control groups reached a mean tumor mass larger than 10% body mass (>2000 mm^3) within less than 2 weeks.

Experiment 4

Experiment 4 demonstrates the inhibitory effect of a combination of intratumoral delivery of specific anti-HER2 "T-Bodies" with concurrent systemic administration of IL-2 on aggressive forms of localized PC tumor progression. Tumors in the control group (receiving 3 direct daily intratumoral injection of 10^7 SP6 chimeric receptor-bearing lymphocytes with concurrent systemic administration of IL-2) had 3-5 times the mean tumor volume compared to the mean tumor volume in the experimental group (Figure 25). The differences in the tumor volume had statistical significance (p=0.03) as well as the differences in the serum PSA levels (p=0.01, Figure 27). In addition tumor growth arrest was evident in the first 3 weeks. FACS analysis of such a tumor that initially responded to the treatment and subsequently regrew, demonstrated a negligible amount of the N29 HER2 epitopes as compared to tumors that were treated with anti-TNP chimeric receptor-bearing lymphocytes (Figure 28). This may indicate the elimination of all available tumor cells that expressed the epitopes to N29 as an accessible target for the specific chimeric receptor-bearing lymphocytes, and that the eventual regrowth of the tumor is the result of outgrowth of antigen negative cells. This hypothesis obviously needs more confirmation, however such a negative

selection is not expected to adversely effect this treatment modality in a clinical setting of neoadjuvant modality for locally or locally advanced PC.

Experiment 5

Unlike the experimentsnts reported above, here we have used WISH-PC14, a novel human prostatic adenocarcinoma xenograft we have recently established (manuscript in preparation) from late recurrent primary tumor (post radiation therapy). Therefore, it uniquely represents a major clinical problem in prostate cancer management: local disease recurence after curative radio-therapy, for which currently the salvage treatment options are very limited. The xenograft over expresses HER2, HER3 and HER4 and secretes PSA to the tumor bearing sera.

The results of experiment 5 (depicted in Fig. 29) demonstrate the inhibitory (and even curative) effect of intra-tumoral administration of anti-HER2 specific chimeric T lymphocytes along with systemic administration of IL2, on another human PC xenograft. The tumors were allowed to grow substentialy s.c as evident by pre treatment serum PSA levels of 8.5 ng/ml. The treatment protocole included 4 daily intratumoral injections of 1.5×10^6 lymphocytes (anti-TNP or anti-HER2) or HBSS alone. All animals were implanted with IL-2 loaded sustained release miniosmotic pumps. The differences in the mean tumor volume (p=0.028) and serum PSA levels (p=0.0075) (Fig. 29) had statistical significance. There were also 55% cure (5 out of 9 mice) in the group treated with anti-HER2 CR bearing cells in comparison to no cure with in both control arms (anti-TNP specific chimeric T lymphocytes and HBSS). In addition, within 51 days of follow-up, no regrowth of palpable tumor could be detected in the mice that were cured.

This experiment models local treatment of prostate cancer tumor reoccurence post radiotherapy. These results suggest that our modality may serve as viable treatment option in local failure of radiotherapy for which there is currently no effective treatment.

Lentiviral based Vectors

HIV-based vectors have been shown to transduce CD34$^+$ cells which have not been pre-stimulated (51). This is consistent with the ability of lentiviral vectors having the ability to transduce non-dividing cells (52). The lentiviral packaging system we are using is also transient based (53). The envelope gene is the VSV G protein (54). The packaging plasmid provides gag, pol and tat. The transfer vector uses the HIV-1 promoter to make viral RNA. It also includes a woodchuck hepatitis virus post-transcriptional regulatory element to enhance expression of the transgene (55). During reverse transcription this promoter is inactivated and an internal PGK or CMV promoter is used (56). The insert containing anti-TNP scFv-CD28-IgEγ with an IRES-GFP was inserted into this vector and it is available for transfection into 293T cells to make virus to infect lymphocytes.

Key Research Accomplishments

• Bacterial expression of PSMA and PSCA

• Mammalian Expression of PSCA-Ig

• Human T lymphocytes transduced with chimeric receptor genes kill a variety of targets in a specific and effective way

• Human T lymphocytes transduced with anti-HER2 receptor prevents human prostate tumor growth in mice.

Reportable Outcomes

Eshhar, Z., Waks, T., Bendavid, A., and Schindler, D.G. Functional expression of chimeric receptor genes in human T cells. J. Immunol. Methods 248, 67-76 (2001)

Pinthus, J. H., Waks, T., Schindler, D. G., Harmelin, A., Said, J. W., Belldegrun, A., Ramon, J. and Eshhar, Z. WISH-PC2: a unique xenograft model of human prostatic small cell carcinoma Cancer Res 60,6563-7 (2000).

Feigelson, W. S., Waks, T., Schindler, D. G., Pinthus, Y., Eisenbach, L., Yarden, Y., and Eshhar, Z.. Two receptor dependence of Neuregulin directed lymphocytes. J. Immunology, In revision 1999.

- Plasmid for bacterial expression of PSMA

- Plasmid for bacterial expression of PSCA

- Plasmid for expression of PSCA-Ig

- Preparation of PSMA protein fragments in bacteria

- Preparation of PSCA in bacteria

- Preparation of PSCA-Ig

- Mice immunized with PSMA

- Mice immunized with PSCA

- Development of bicistronic retrovector producing chimeric receptor and GFP

- Packaging cell line to produce retrovectors transducing anti-TNP chimeric receptors

- Packaging cell line to produce retrovectors transducing anti-HER2 chimeric receptors

- Packaging cell line to produce retrovectors transducing NDF specific chimeric receptors

Conclusions:

I. In order to make antibodies to prostate tumor antigens, recombinant fusion PSMA and PSCA proteins have been produced and found immunogenic in mice.

II. A retroviral transduction system has been set up to enable efficient transfer of functional chimeric receptor genes into human T cells

III. HER2 specific chimeric receptor expressing effector lymphocytes can prevent the growth of human xenografts in mice.

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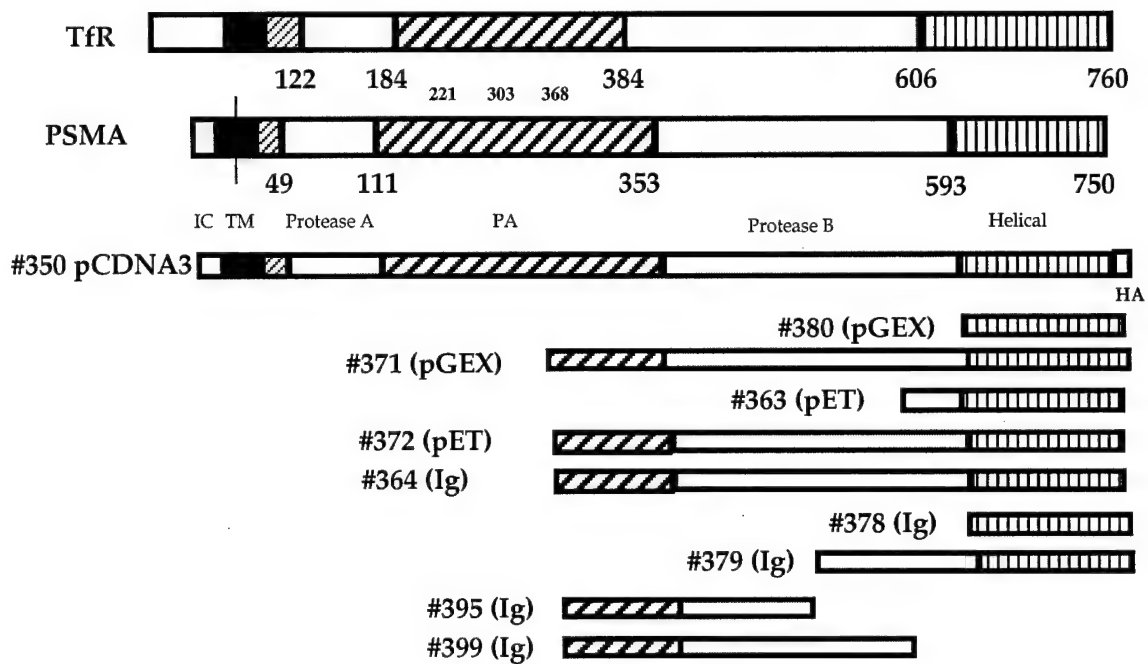
Figure 1. PSA compared to transferrin receptor and fragments in expression vectors.
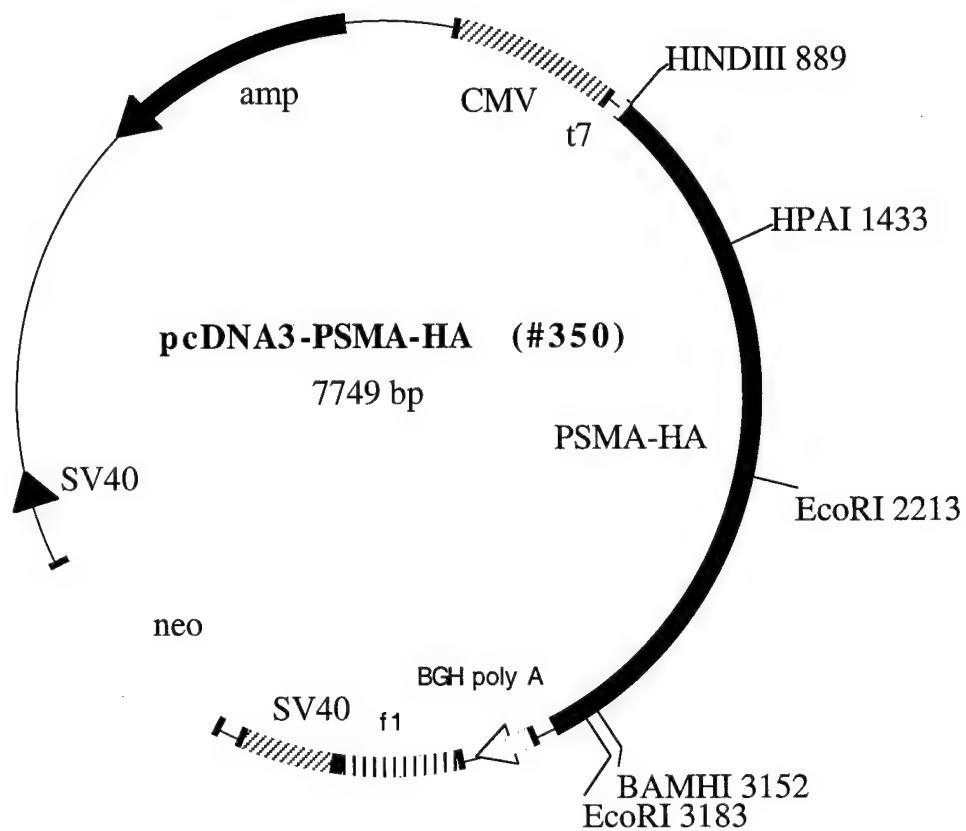
Figure 2- Schematic map of the plasmid for the expression of PSMA with a HA tag. A CMV IE promoter drives expression and the plasmid has a bacterial *neo* gene for selection in mammalian cells by G-418.
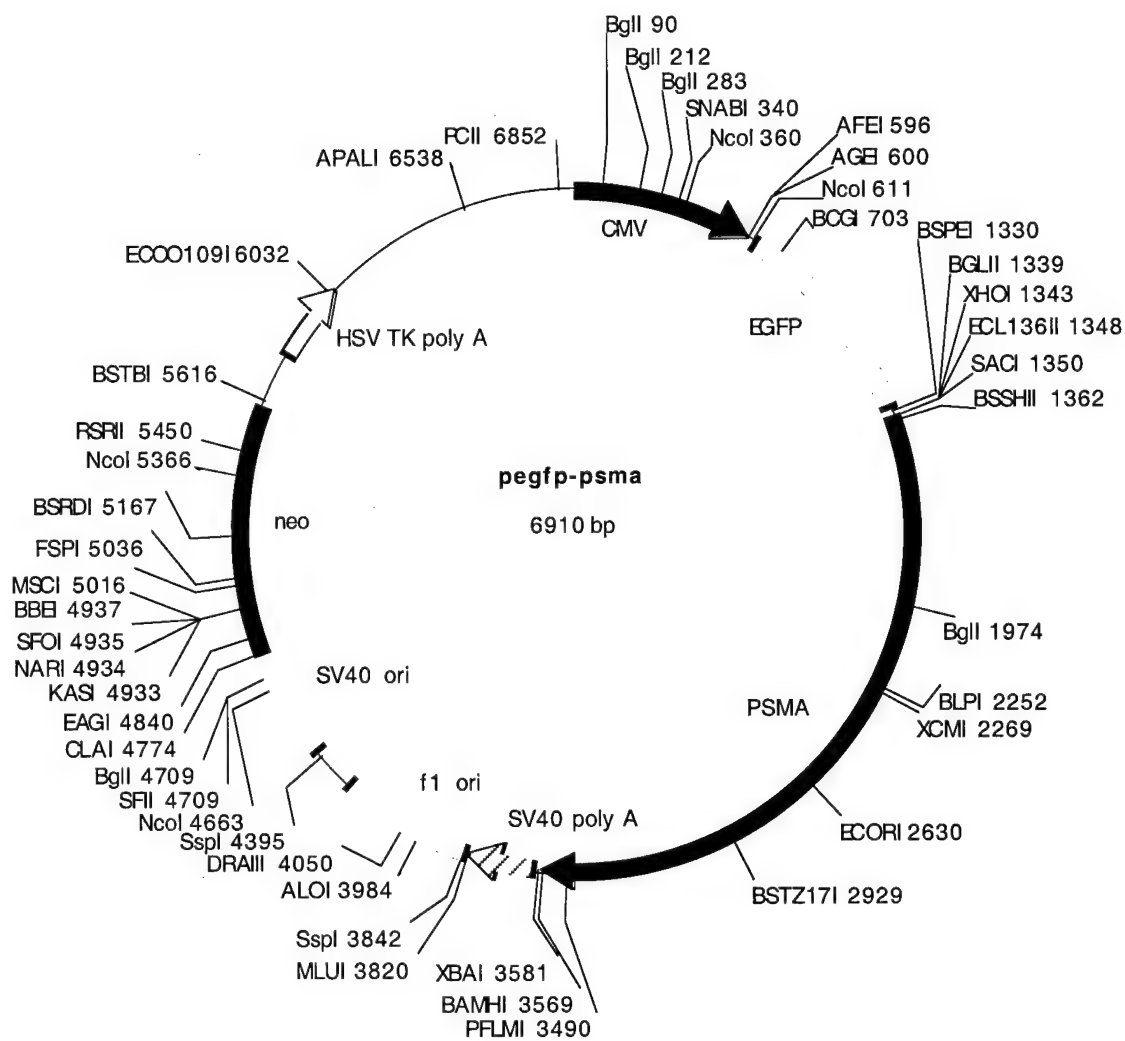
Figure 3. Plasmid Map of Expression Vector of EGFP-PSMA fusion protein.
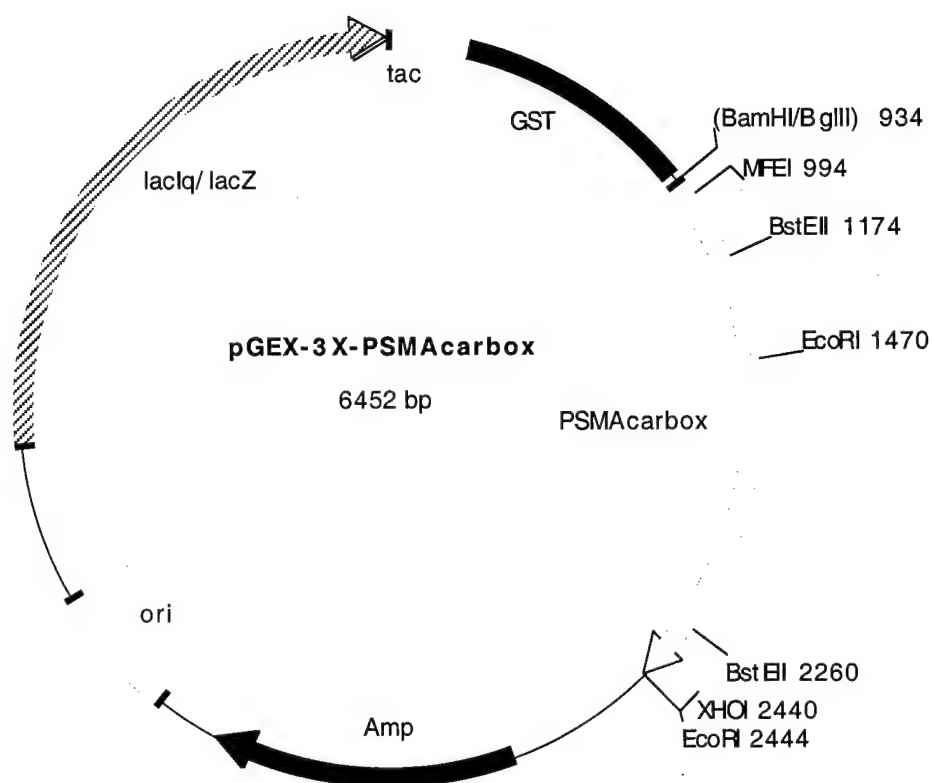
Figure 4- Schematic map of a pGEX plasmid for the bacterial expression of PSMA fragments. The gene lacI is the repressor for the hybrid trp-lac (tac) promoter. GST is glutathione-S-transferase. The piece is cloned between a BamH I site and an Eco I site.
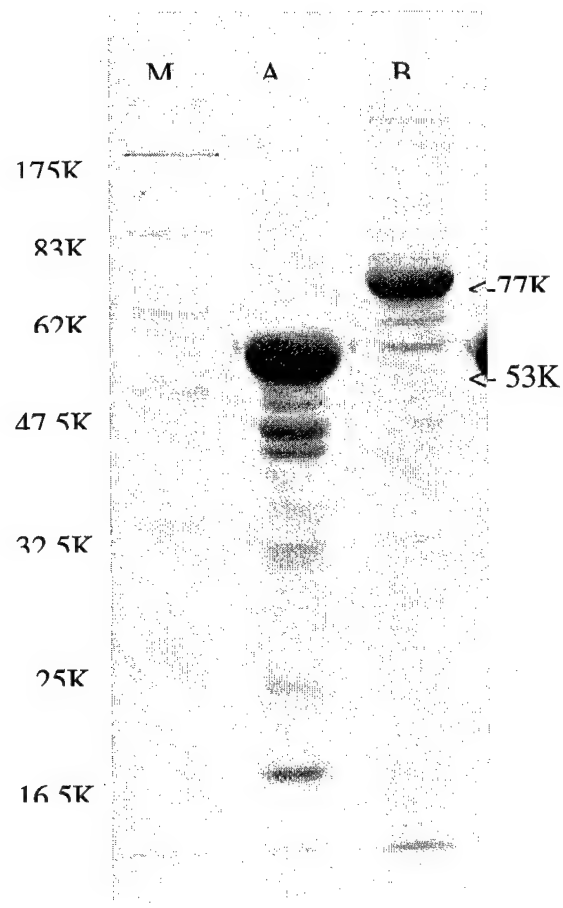
Figure 5- SDS PAGE of refolded GST-PA Domain (A) and IMPACT-Helical Domain(B)
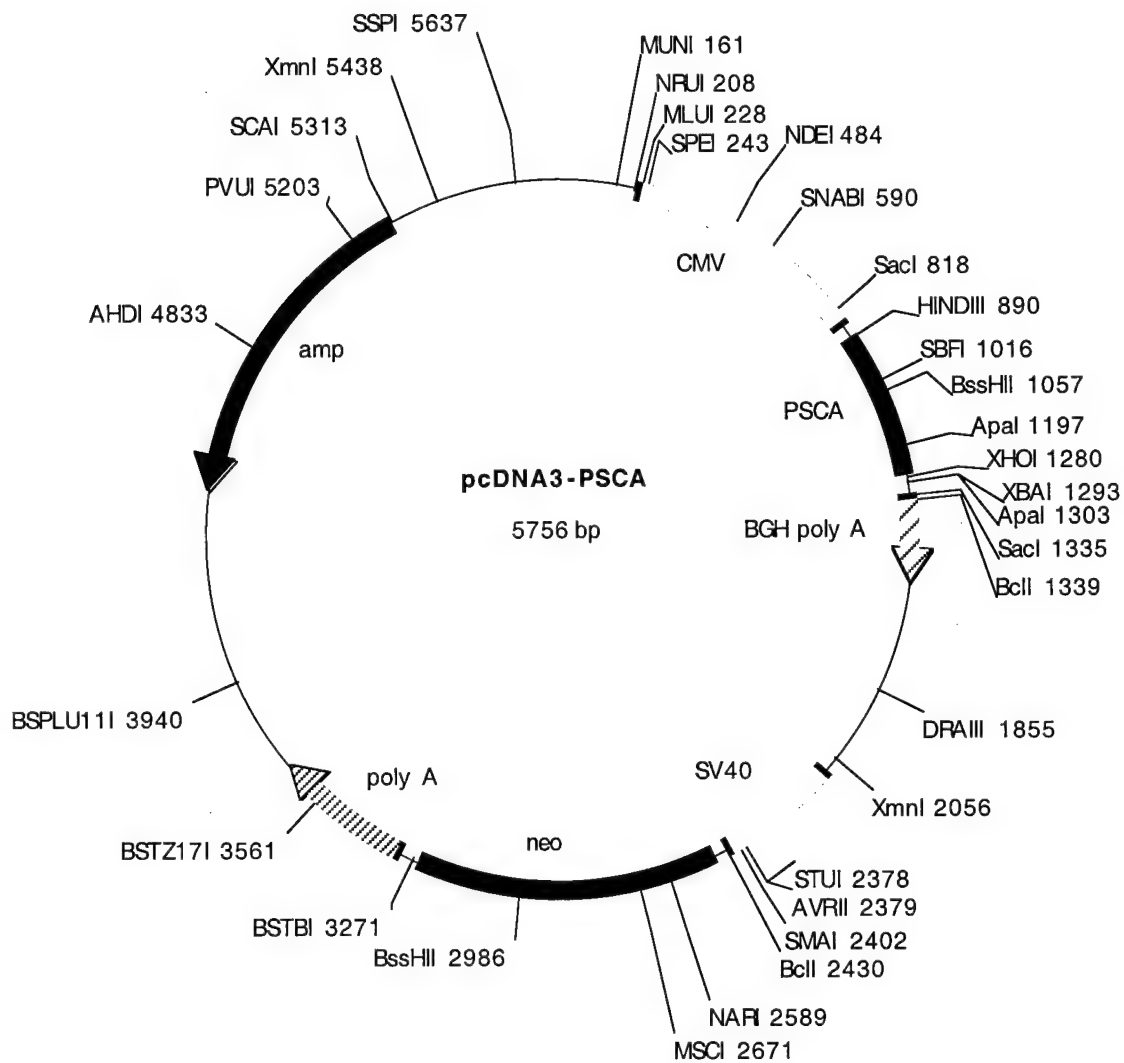
Figure 6- Schematic map of mammalian expression vector for PSCA. It is similar to the plasmid in figure 2. The cloning sites are the Hind III site at the 3' end and the Xho I site at the 5' end.

35
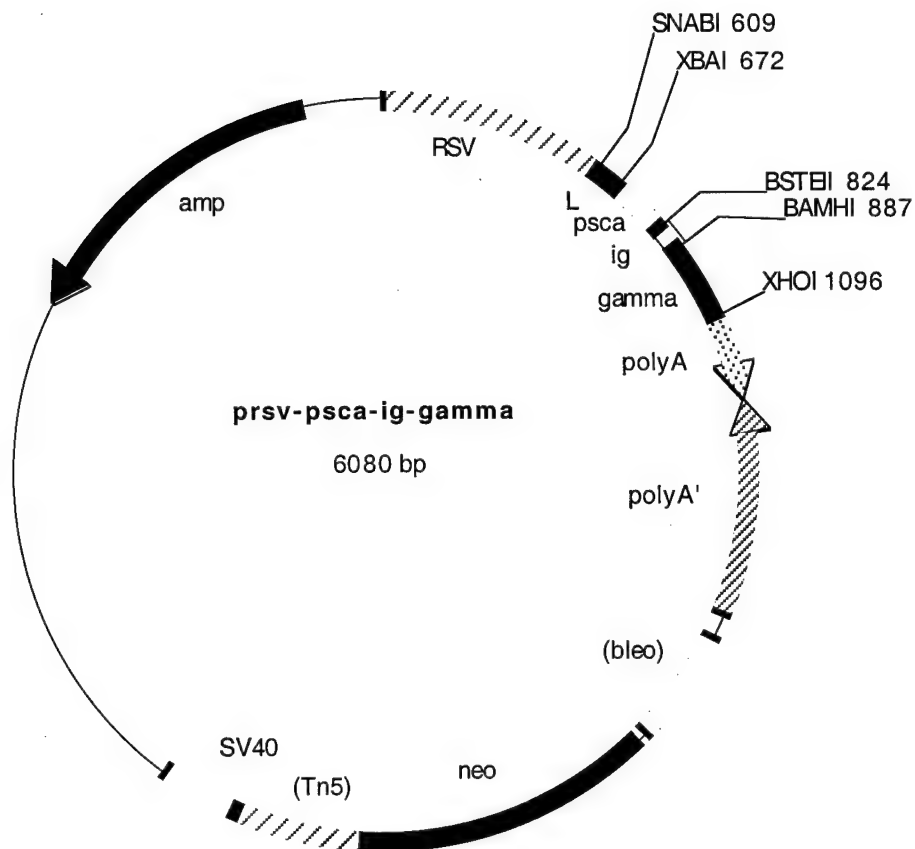
Figure 7- Schematic map of mammalian expression vector for surface expressed PSCA. A RSV LTR drives the expression and the plasmid has a bacterial *neo* gene for selection in mammalian cells by G-418. The mature PSCA protein is encoded by the sequence between the Xba I and the BstE II sites. The Ig portion serves as a spacer from the cell membrane and the gamma portion is from the Fcε receptor gamma chain and provides a
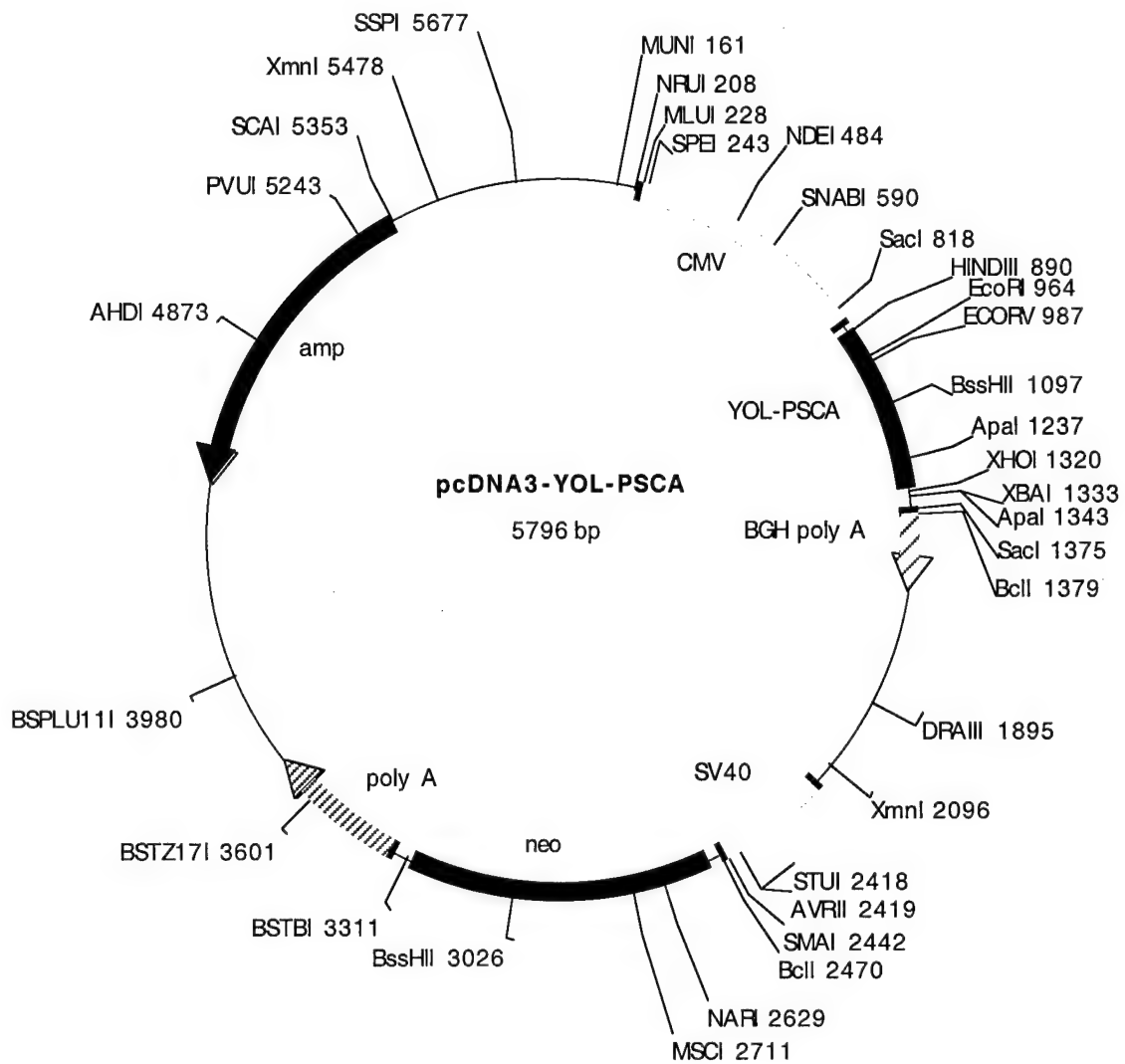
Figure 8- Schematic map of mammalian expression vector for surface expressed PSCA with a YOL tag for antibody detection. It is similar to the plasmid in figure 2.
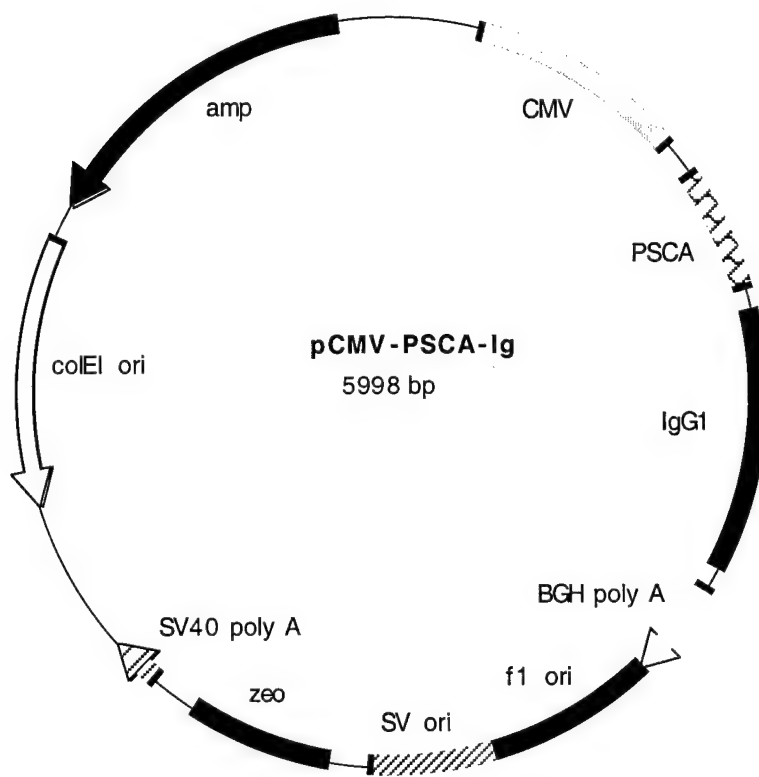
Figure 9- Schematic map of mammalian expression vector for the secreted PSCA-Ig fusion. The plasmid contains the *zeo* gene for selection with bleomycin derivatives.
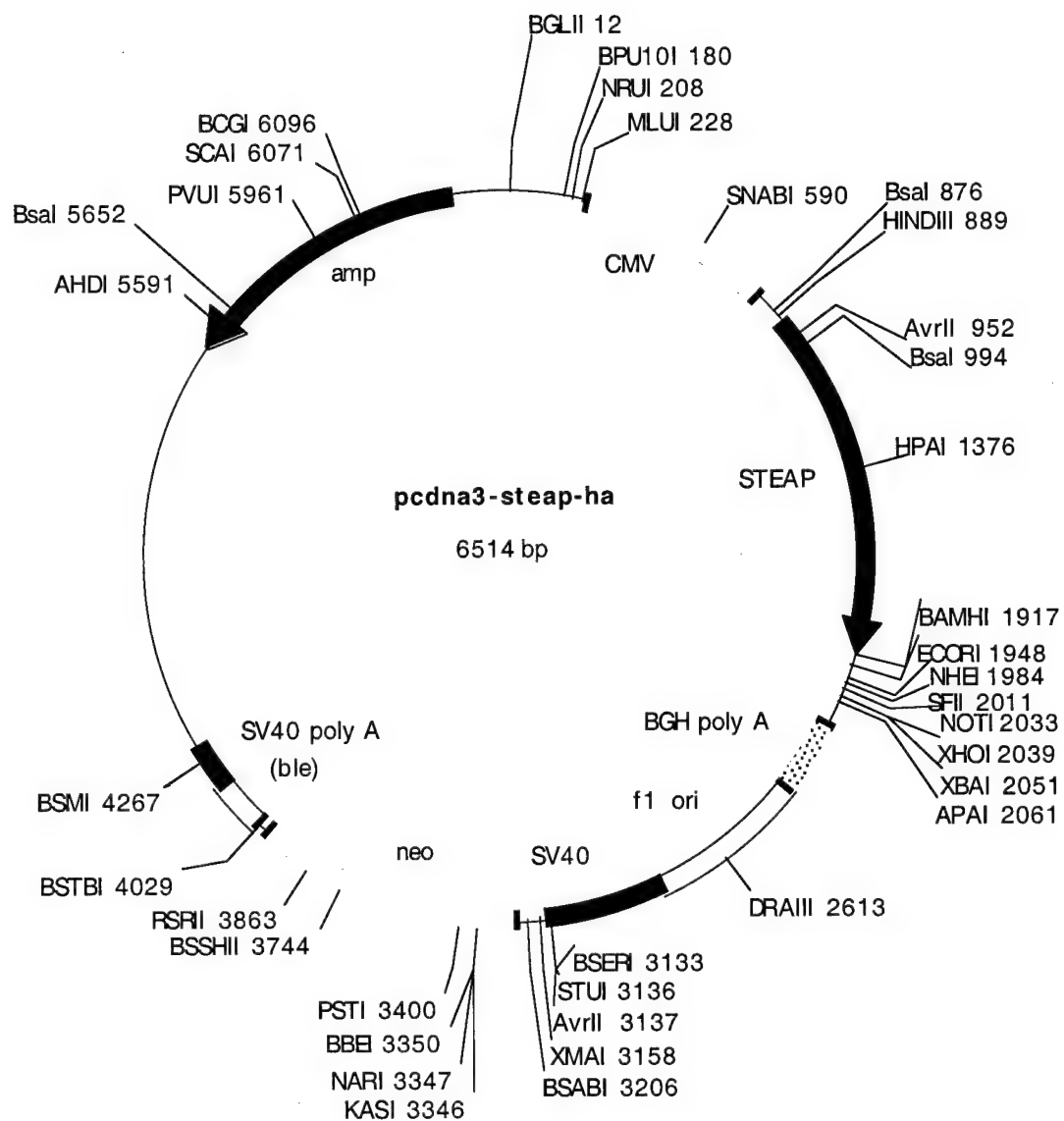
Figure 10- Map of pCDNA3-STEAP-HA vector
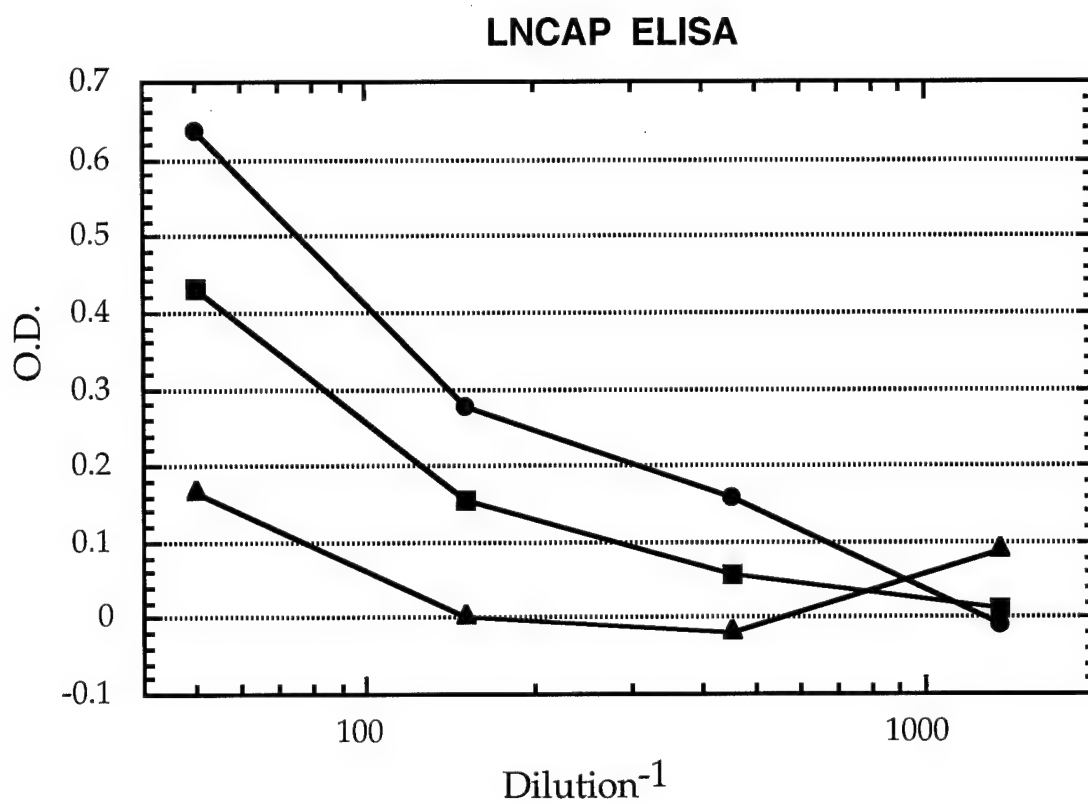
Figure 11-Testing immunized mouse sera on LNCaP cells with a whole cell ELISA. Circles are using sera PSMAII3, squares are from PSCA5, and triangles are from normal mouse sera. Values obtained using PBS have been subtracted.

pBULLET-SP6-CD28-γ-IRES-GFP
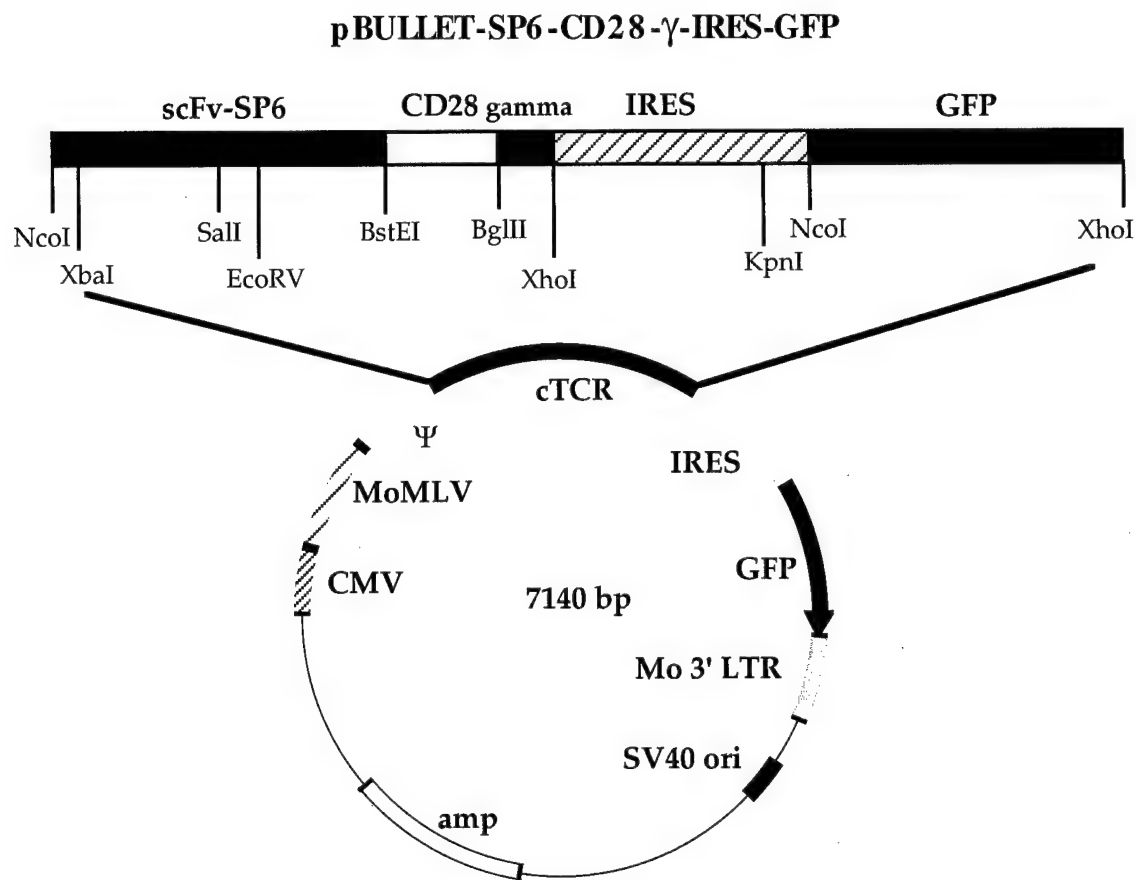
Figure 12- Schematic map of the retroviral transfer vector.

GFP-Staining

Biotin anti -SP-6 id

GFP-Staining
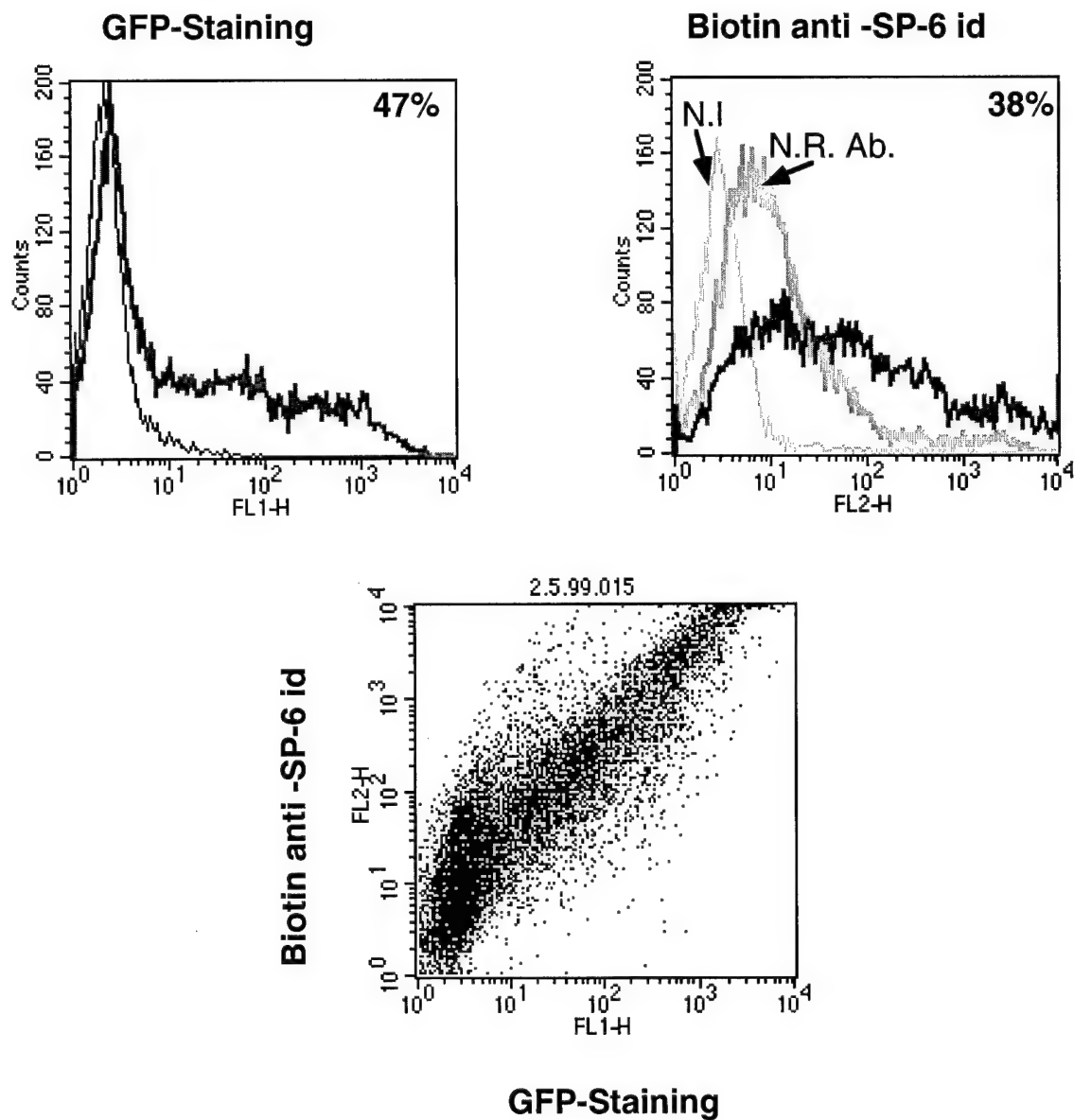
Figure 13- FACS analysis of infected human lymphocytes. The top left hand panel shows the transduction of the GFP gene by measuring fluorescence and the top right hand shows the transduction of the chimeric receptor by measuring the amount of receptor with biotinylated monoclonal antibody specific for the scFv. The receptor was stained with phycoerythrin-conjugated streptavidin. There are two negative controls. One is non-infected lymphocytes (N.I.) and the other is stained with a non-relevant antibody instead of the anti-scFv. The
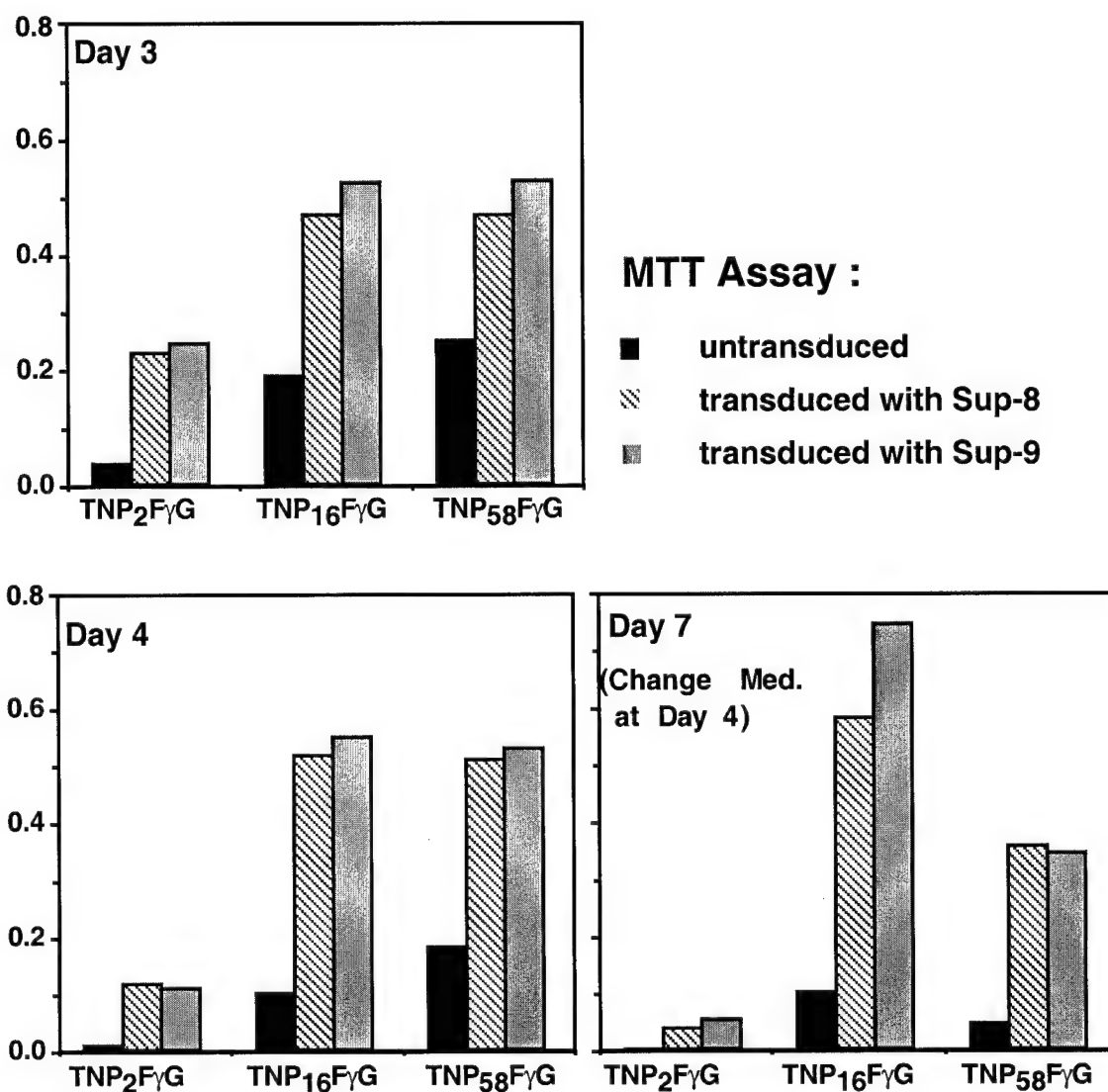
Figure 14- Infected human lymphocytes were grown on fowl immunoglobulin gamma globulin containing different loadings of TNP. The number of live cells was measured by an MTT assay.
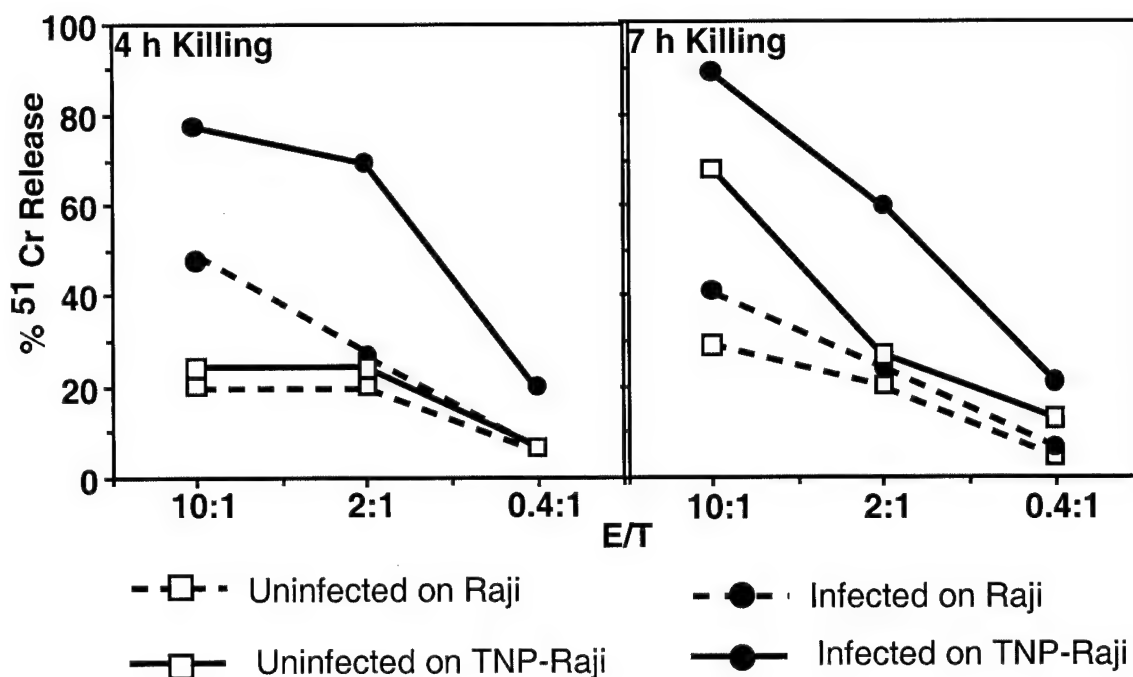
Figure 15- Killing of Raji target cells with human lymphocytes transduced with anti-TNP scFv receptor. The panel on the left measures killing by release of 51Cr after 4 hours and the one on the right after 7 hours. Dotted lines used unmodified Raji as the target and solid lines used TNP-ylated Raji as a target. Open squares are the results from uninfected human lymphocytes and filled circles use infected lymphocytes. Effector cell to target cell ratios varied from 0.4 to 10.
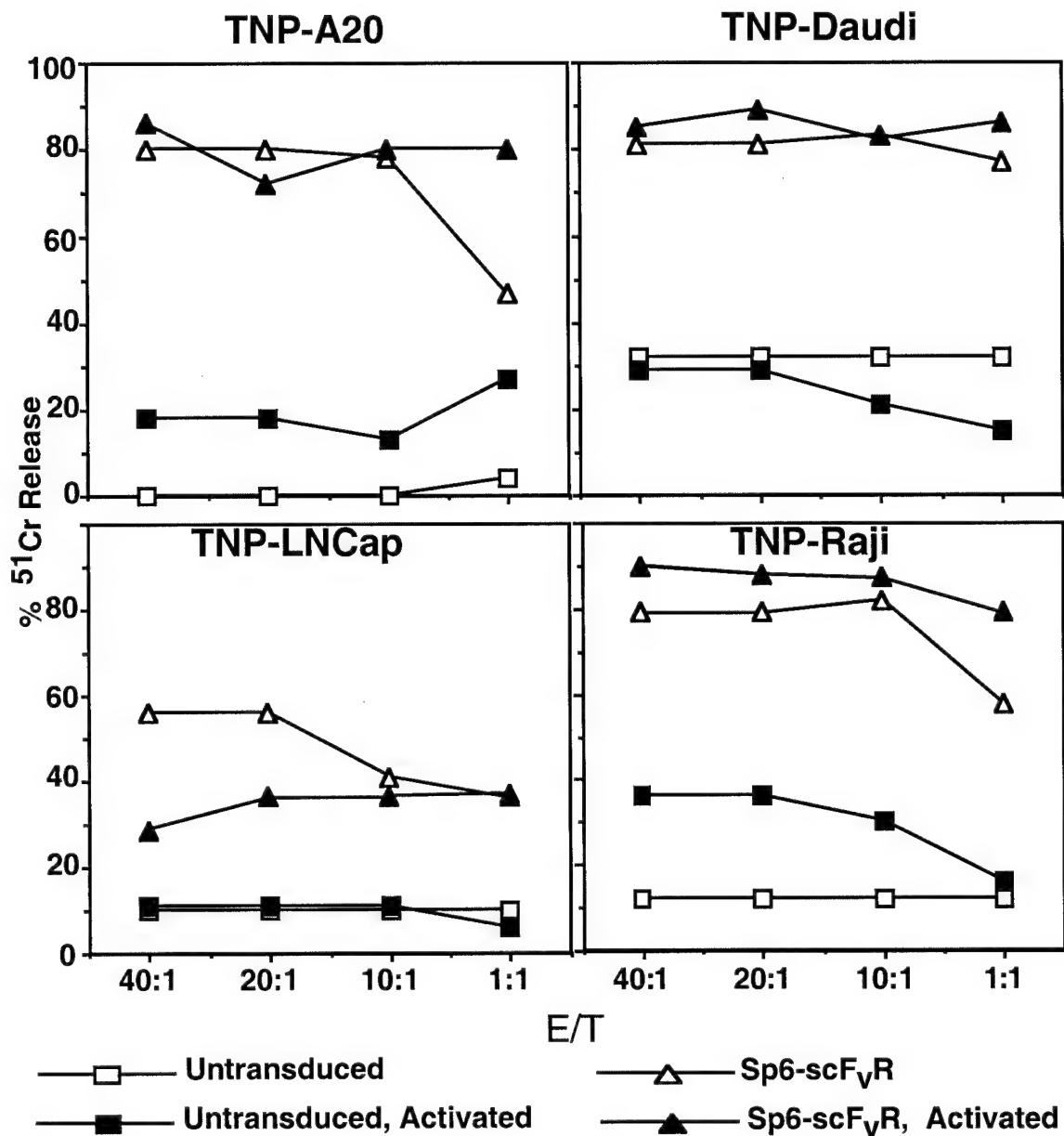
Figure 16- Specific killing of TNP-ylated target cells by human lymphocytes bearing anti-TNP scFv chimeric receptor. Squares are uninfected lymphocytes and triangles are infected lymphocytes. Solid symbols represent killing by activated human lymphocytes.

Killing of Target Cells by Transduced Lympocytes
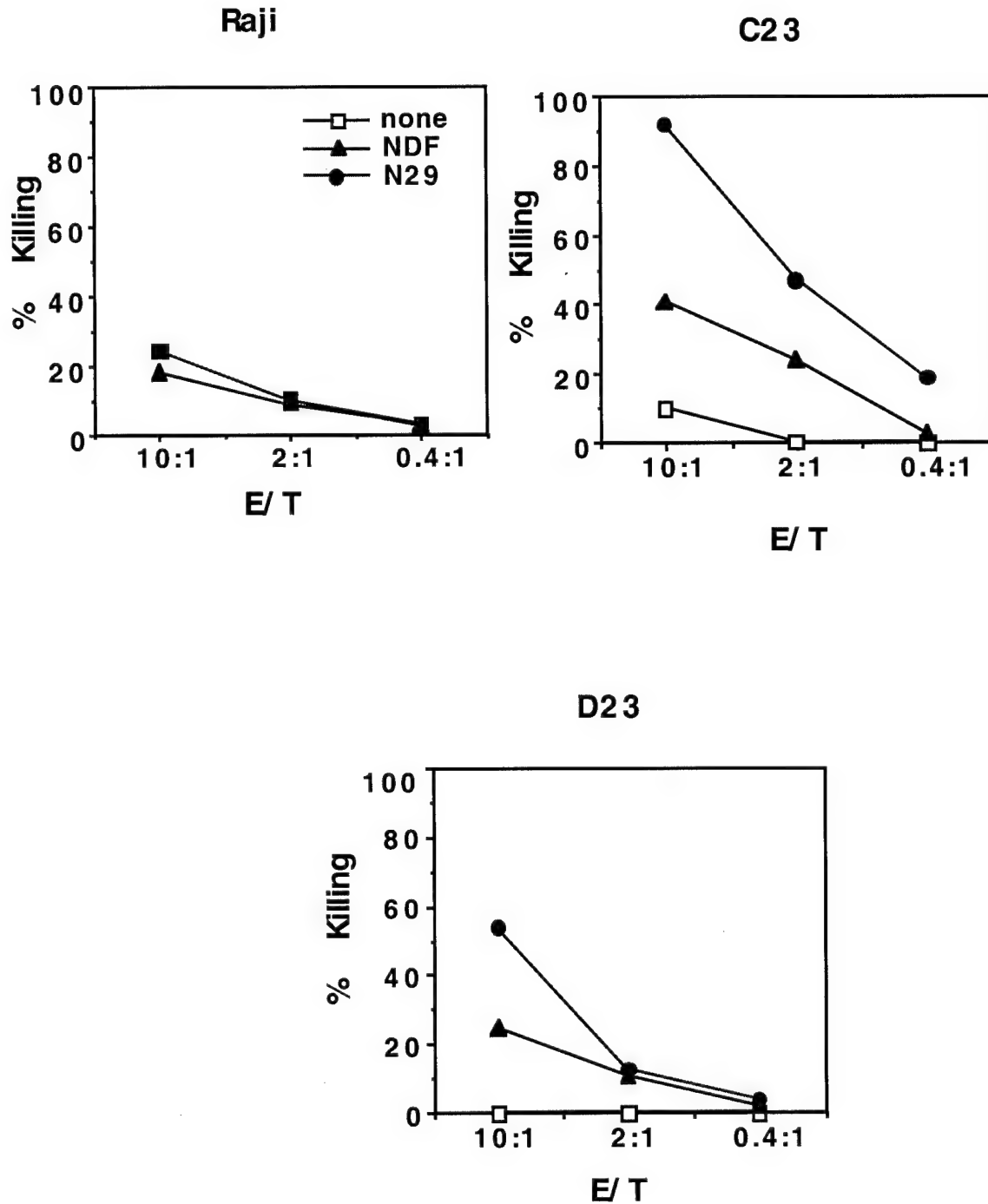
Figure 17- Killing of Target Cells by Transduced Lymphocytes
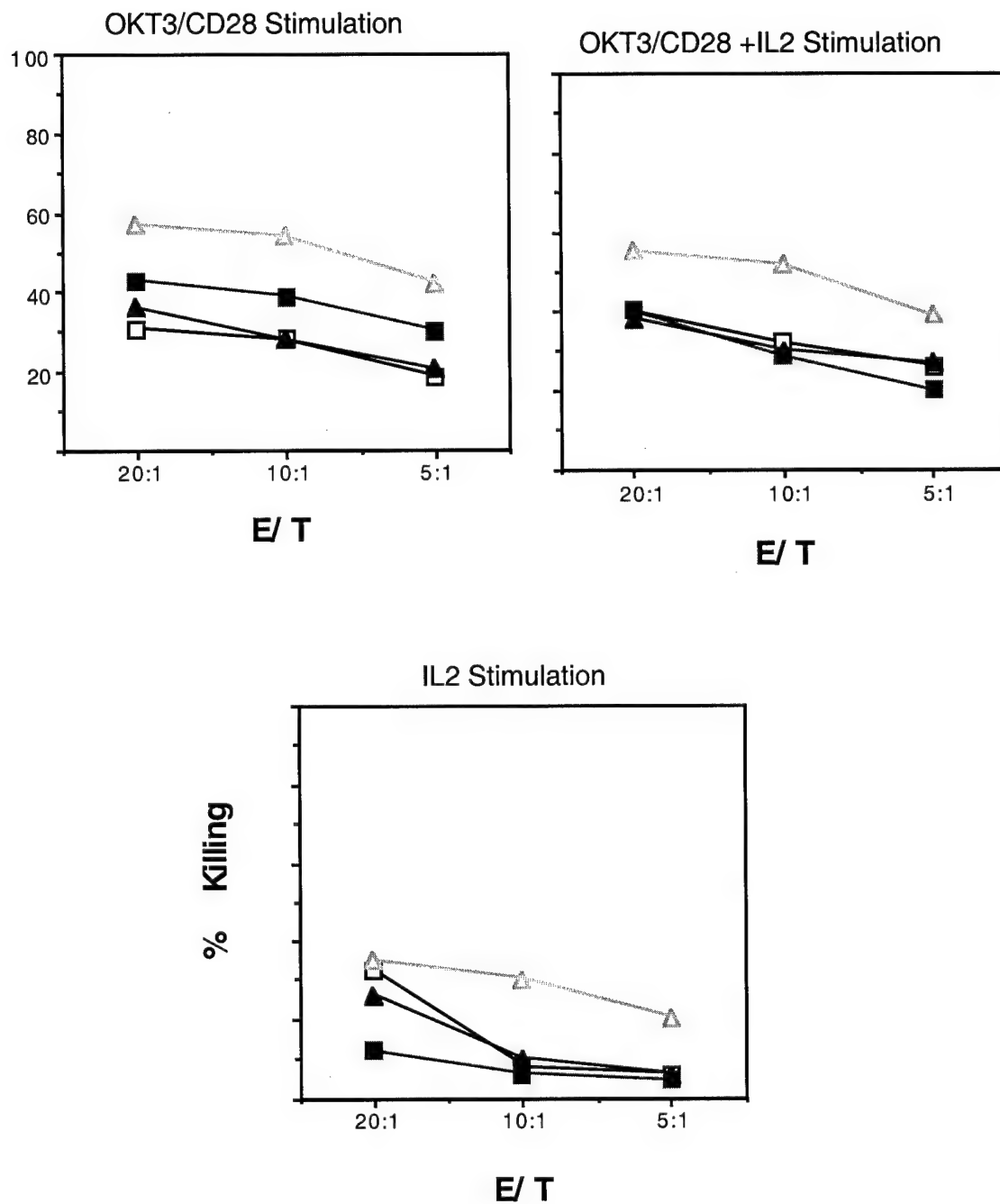
Killing of C23 Target Cells

Figure 18- Killing of CHO cells expressing HER2 and HER3

Killing of D23 Target Cells by Transduced Lympocytes
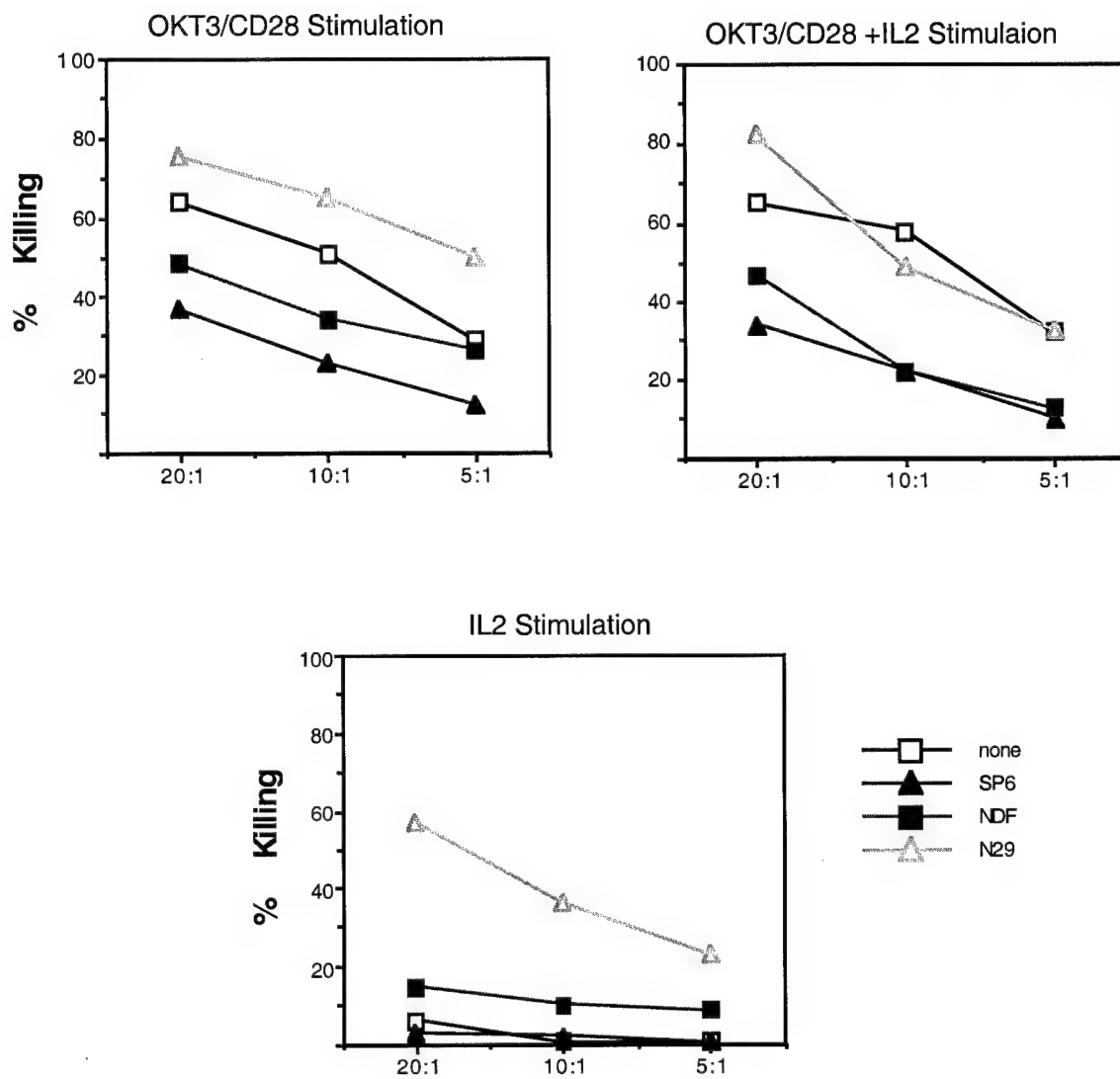
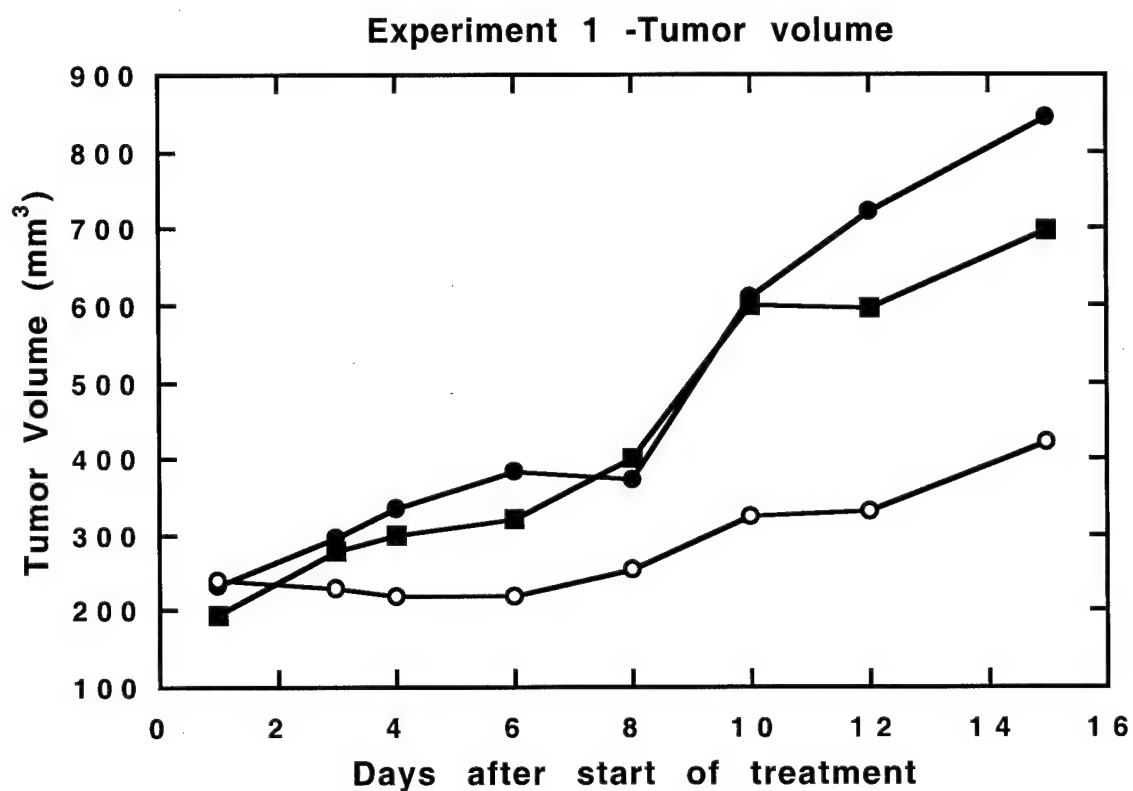**Figure 19.** Killing of D23 Target Cells by Transduced Lymphocytes

Experiment 1 -Tumor volume

Figure 20. Effect of chimeric receptor bearing lymphocytes on tumor.

Open circles -tumor injected with buffer

Closed circles- tumor injected with lymphocytes bearing an irrelevant receptor.

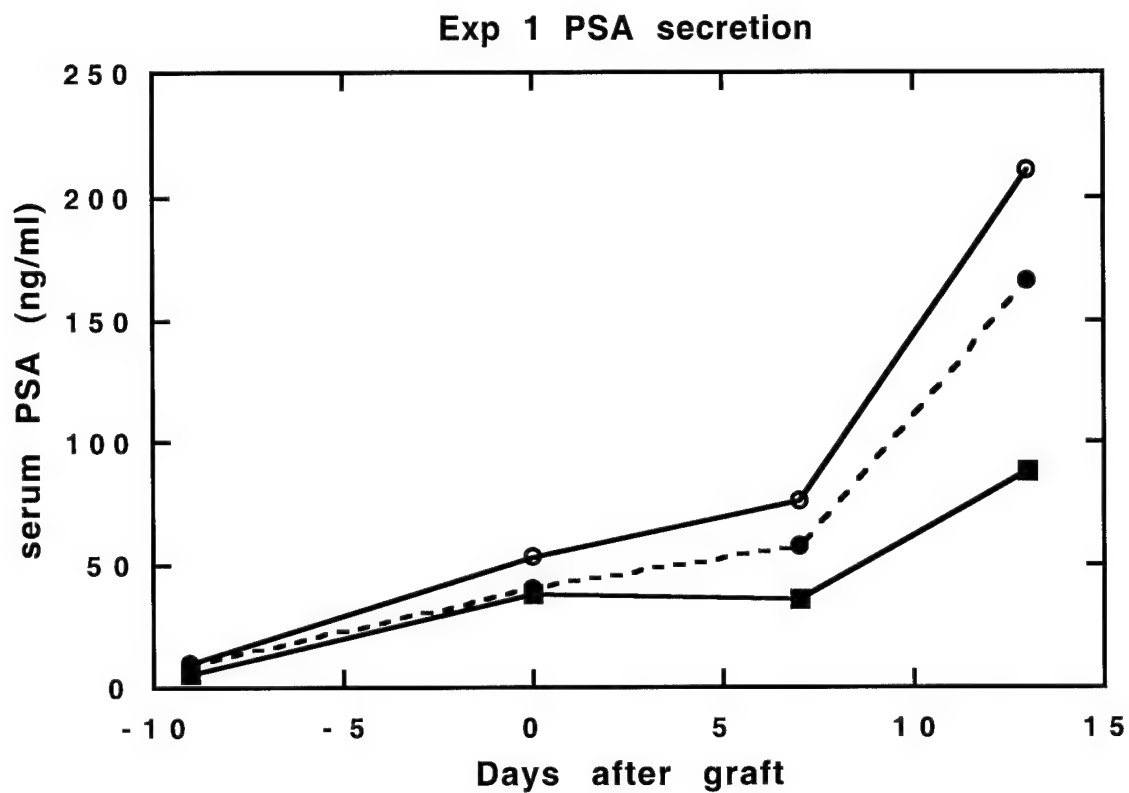Closed squares- tumor injected with lymphocytes bearing a HER2 directing receptor.

Figure 21- Effect of transduced lymphocytes on PSA secretion.

Treatment was started on Day 0.

Open circles -tumor injected with buffer

Closed circles- tumor injected with lymphocytes bearing an irrelevant receptor.

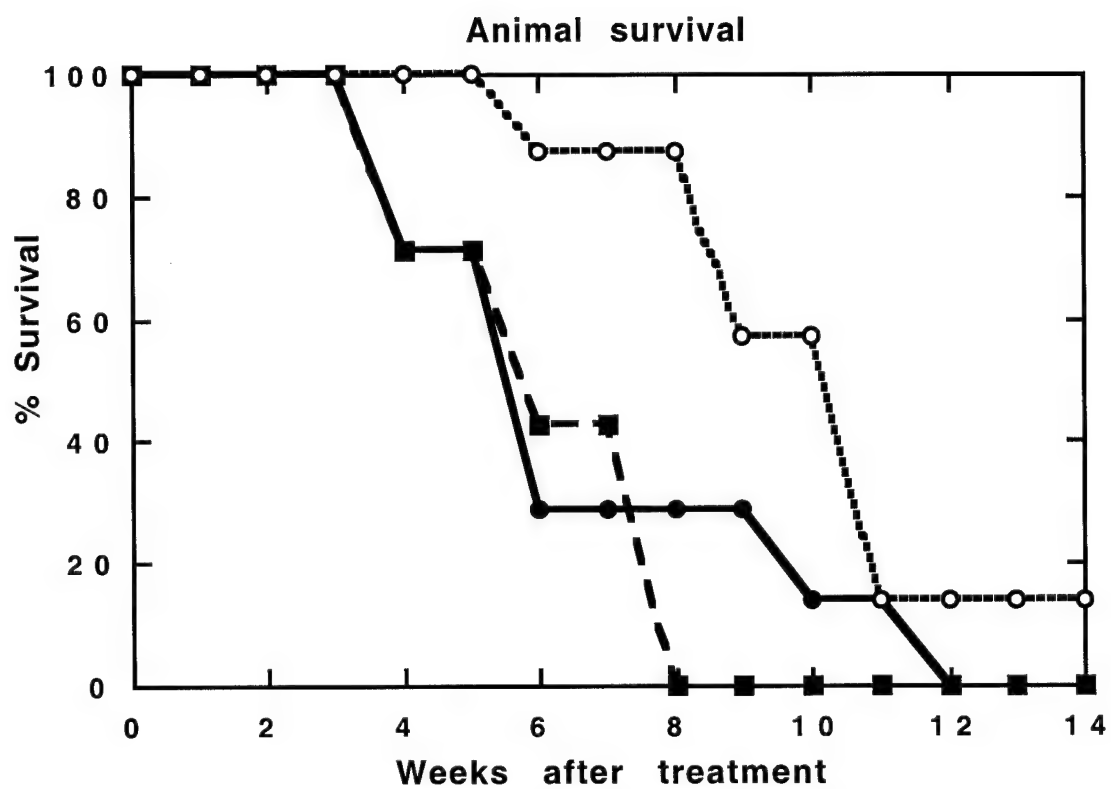Closed squares- tumor injected with lymphocytes bearing a HER2 directing receptor

Figure 22 - Survival after treatment with anti-HER2 chimeric receptor lymphocytes.

　　　　Open circles - HER2
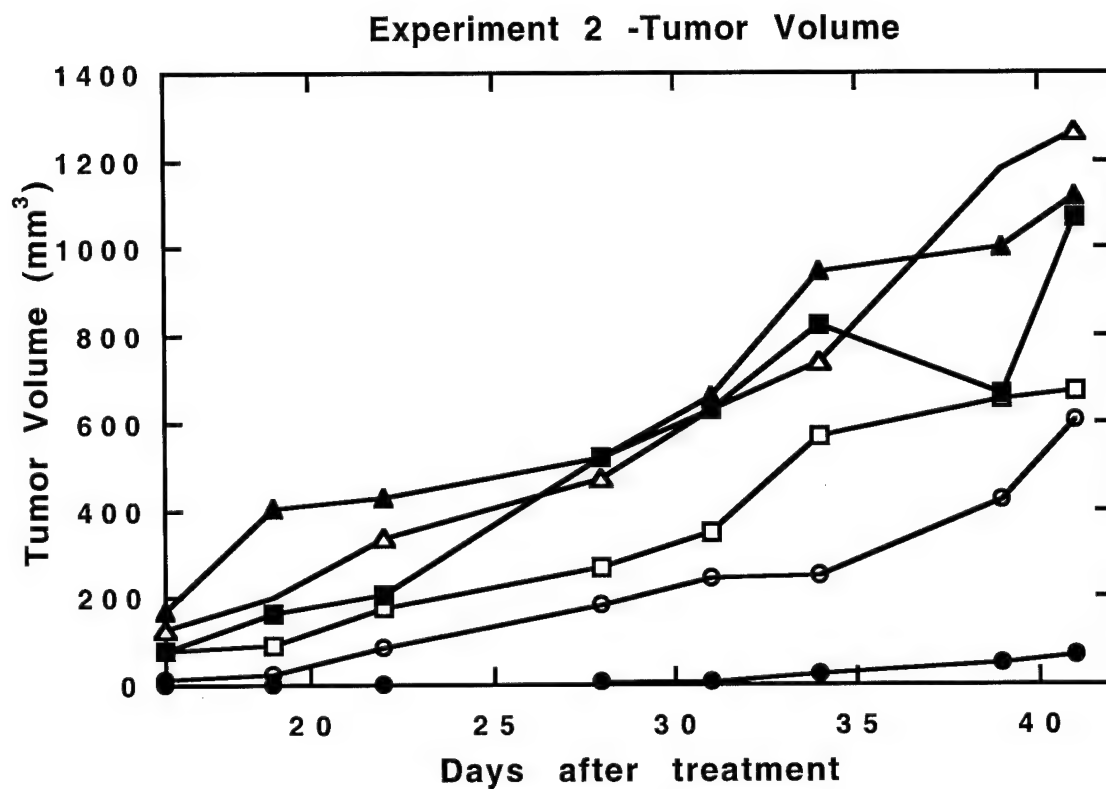
　　　　Squares- TNP

　　　　Closed circles- Buffer

Experiment 2 - Tumor Volume

Figure 23- Closed symbols are with IL-2, Open symbols without.
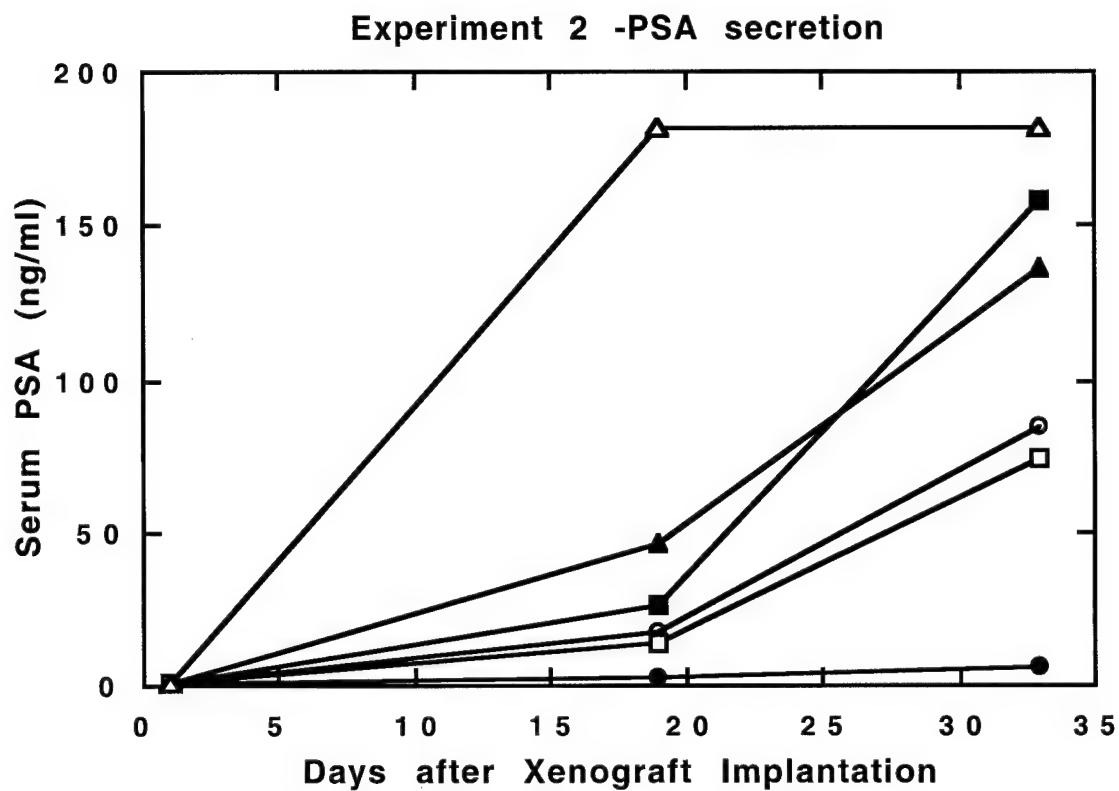Triangles TNP; Squares- Buffer; Circles- HER2

Figure 24- Results of Experiment 2 on PSA secretion
Open symbols are sham treated.
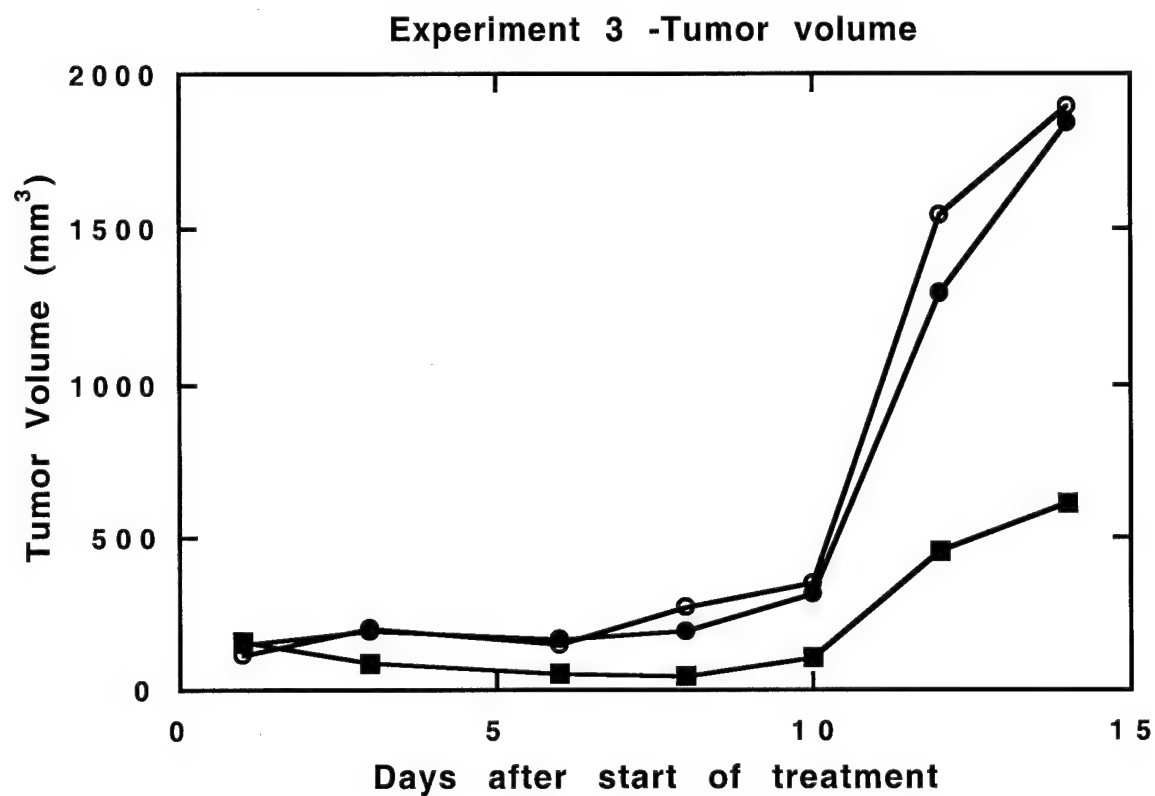Circle-HER2, Squares- Buffer, Triangles-TNP

Figure 25-Effect of chimeric receptor bearing lymphocytes on tumor volume in Experiment 3.

Treatment is intratumoral injections of lymphocytes with IL-2 treatment.

Open circles -tumor injected with buffer

Closed circles- tumor injected with lymphocytes bearing an irrelevant receptor.
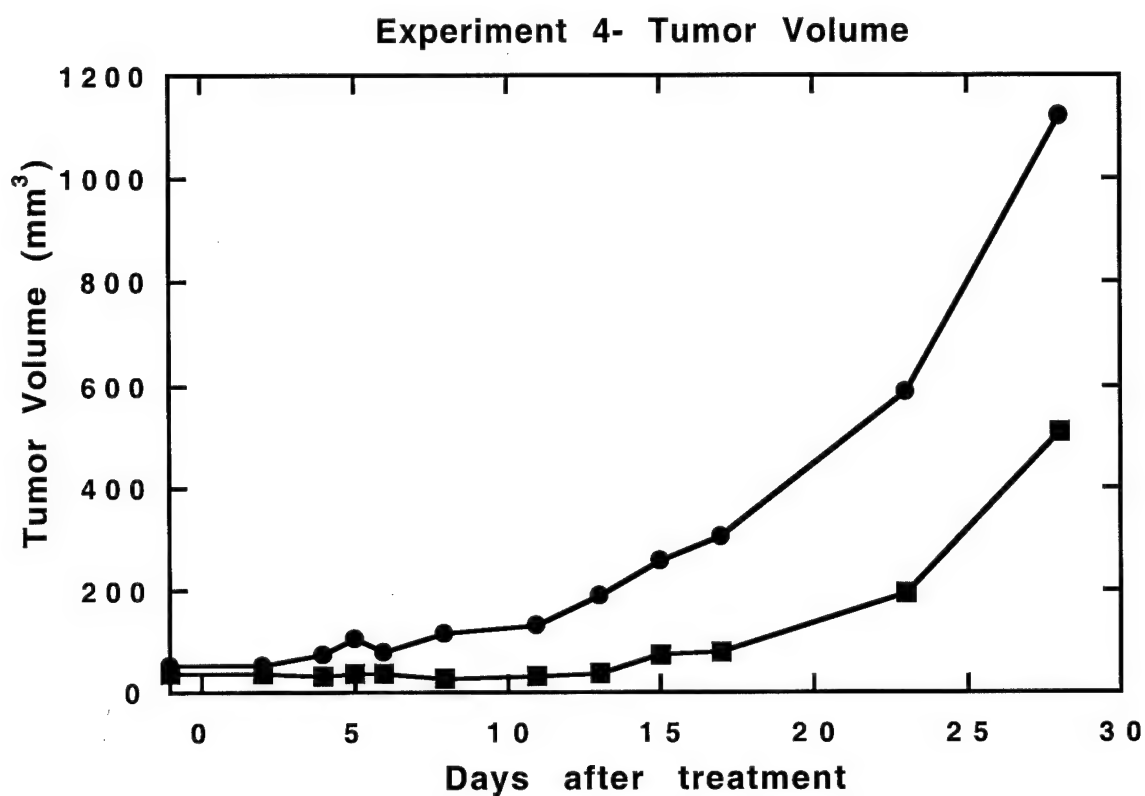
Figure 26- Effect of chimeric receptor bearing lymphocytes on tumor volume in Experiment 4. Circles-TNP, Squares-HER2
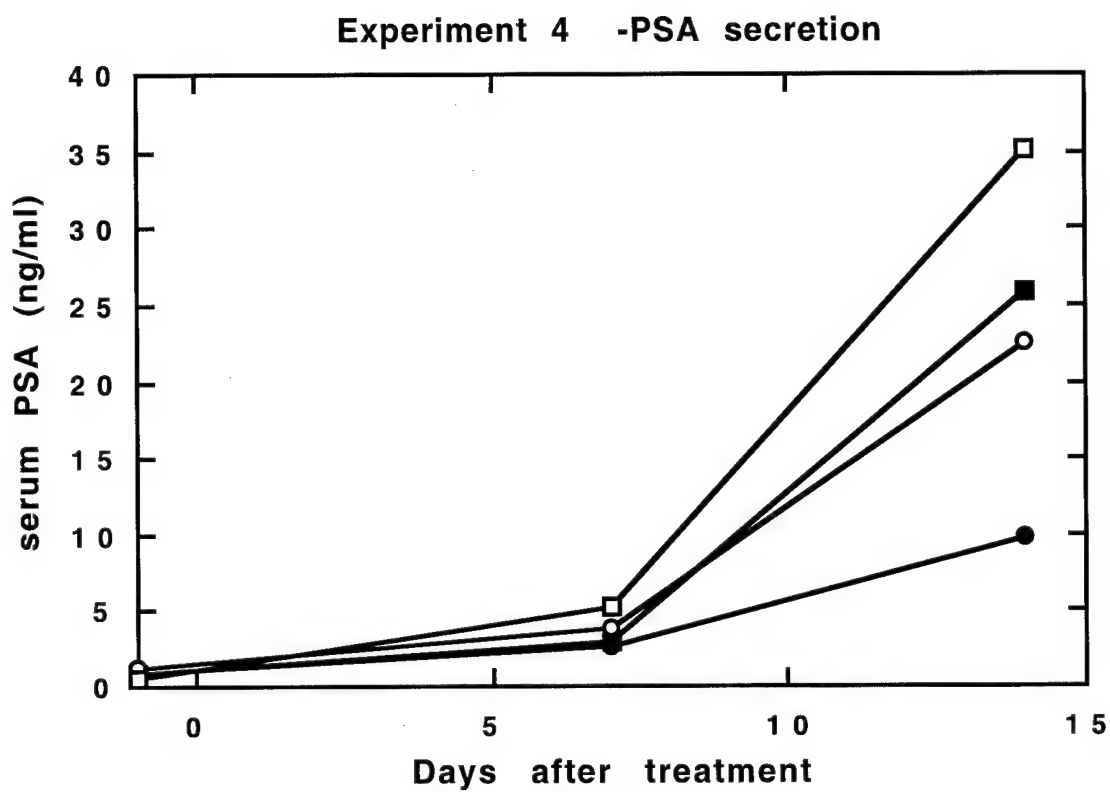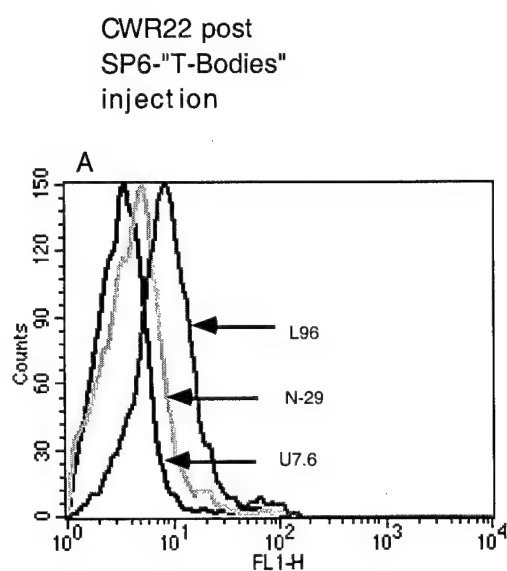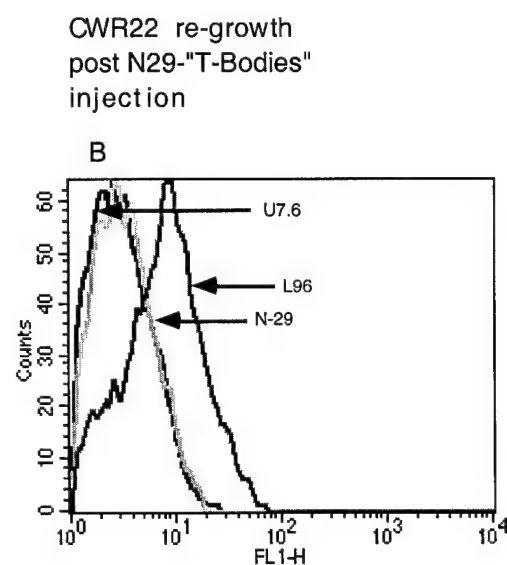
Figure 27- Effect of chimeric receptor bearing lymphocytes on PSA secretion in Experiment 4.
Circles HER2; Squares TNP
Closed symbols - Treated with IL-2;Open symbols no IL-2 treatment

Figure 28. Loss of N29 HER2 epitope but retention of L96 HER2 epitope on treated tumors.

Effect of Intratumoral Injection of anti-HER2 Chimeric Lymphocytes on Human Prostate Cancer Xenografts (WISH-PC14)
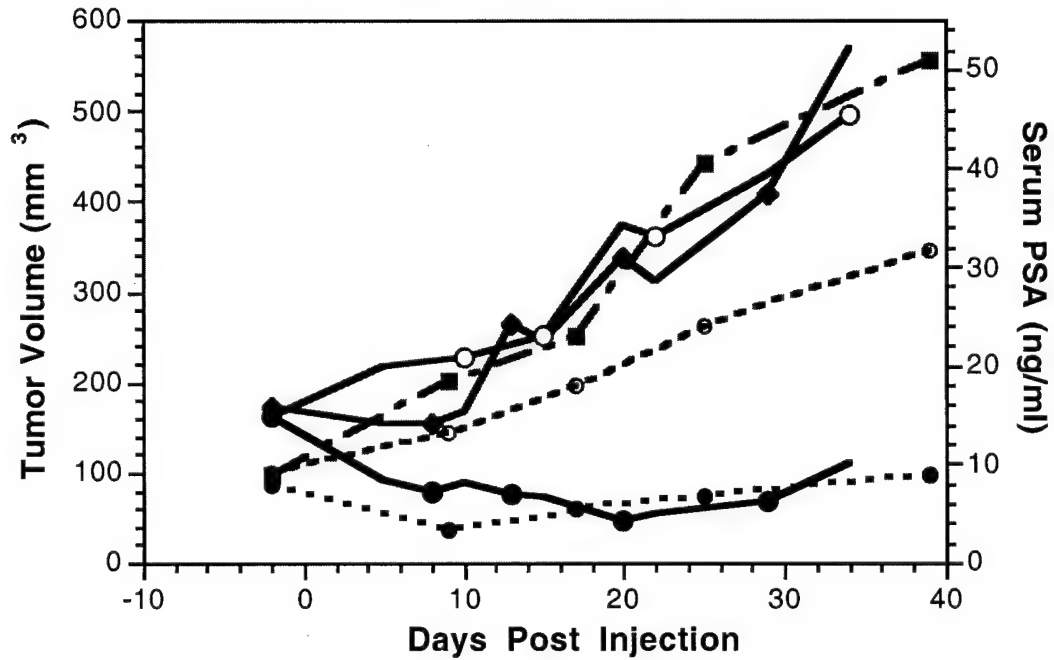
Figure 29. Effect of chimeric receptor bearing lymphocytes on the WISH-PC14 human prostate cancer xenograft: Tumor volume (smooth line) and PSA secretion (dashed line).

Treatment is intratumoral injection of lymphocytes with systemic IL-2 treatment.

Squares: tumor injected with buffer.

Open circles- tumor injected with lymphocytes bearing an irrelevant receptor.

Closed circles: tumor injected with lymphocytes bearing a HER2 directing receptor.

Table 1. List of Primers
Cloning PSMA

#26641 5' GCG<u>AAGCTT</u>GCGCCGAGATGTGGAATCTCCTTCACG

#26642 5' CTT<u>GAATTC</u>TCCTCTGCCCACTCAGTAGAACCAAGAAG

#26643 5' GGA<u>GAATTC</u>AAGACTCCTTCAAGAGCGTGGCGTGGCTTA

#26644 5' CGC<u>GGATCC</u>GCTACTTCACTCAAAGTCTCTGCAGCTGCCTGC

Expression of PSMA in pGEX

#29076 G<u>GGATCC</u>ATTATGCTGTAGTTTTAAGAAAGTATGCTGACAAAATCTAC

#27910 GC<u>GAATTC</u>G<u>CTCGAG</u>TGGCTACTTCACTCAAAGTCTCTGC

#27909 5'

GC<u>AGATCT</u>CC<u>GGATCC</u>GCAAATGAATATGCTTATAGGCGTGGAATTG

Expression of PSMA in IMPACT

#44404 5' GC<u>GTCGAC</u>CTCACTGTGGCCCAGGTTCGAGG

#44405 5' GC<u>GAATTC</u>GTCCACTTTGCTTTCAATATCAAACAG

Cloning of PSCA

#26586 5' CGC<u>AAGCTT</u>AGGCAGTGACCATGAAGGCTGTGCTGC

#26587 5' CCG<u>CTCGAG</u>CCTATAGCTGGCCGGGTCCCCAGAGC

Mammalian expression of PSCA

#27190 5' GC<u>TCTAGA</u>CTGCAGCCAGGCACTGCCCTGCT

#27191 5' GC<u>GGTGACC</u>ACGGCATGGGCCCCGCTGGCGTTG

#27237 5'

GC<u>GAATTC</u>TCCGAGGCCCGTGAGGATATCCTGCAGCCAGGCACTGCCCTG

59

#27186 GC<u>GAATTC</u>ACCCTCCTCGGCCAAGCCTGCCATCA

Construction of PSCA-Ig

#27456 5' GC<u>GGATCC</u>GCAGCAGCCGGCTGCAGGGCATGGGC

Bacterial expression of PSCA

#28254 5' CGC<u>GGATCC</u>CCGATATCCTGCAGCCAGGCACTGCCCTGCT

#27748 5' GC<u>GAATTC</u>GCAGCCGGCTGCAGGGCATGGGCCCCGC

Restriction sites are underlined.

Table 2.

GFP Expression in Human PBL Infected with Supernatants of Different Packaging Cells

Packaging Cells	48h		5 days	
	A	B	A	B
PA-317.7	<1	1	30	10
PG-13.8	10	40	30	50
PG-13.9	30	30	30	40
PG-13.13	1	15	40	50
FLY-GA16.1	0	0	5	1-5
FLY-RD18.4	0	0	>1	0

A : Infection according to K. E. Pollok et al.

J. of Virology, 72; 4882, 1998.

B : Infection according to M. Weijtens et al.

Gene Therapy, 5; 1195, 1998

Numbers are % infected cells as measured by fluorescence

Table III.

Experiment	Mean Initial PSA (ng/ml)	Initial Tumor Burden Mean volume (mm³)
1	8.6	220
2	1.24	70
3	18.6	140
4	0.9	50

Table. IV

Experiment	Construct	Transgene expression	Remarks
1	SP6	48% (45-54%)	Frozen lymphocytes
1	N29	29.4% (27-32%)	Frozen lymphocytes
2	SP6	57% (45-61%)	Fresh lymphocytes
2	N29	63% (56-70%)	Fresh lymphocytes
3	SP6	61.5%	Fresh lymphocytes
3	N29	72%	Fresh lymphocytes
3	SP6	40% (36-44%)	Frozen transduced lymphocytes (from exp.2)
3	N29	47.5% (43-52%)	Frozen transduced lymphocytes (from exp.2)
4	SP6	55.6% (51-59%)	Fresh lymphocytes
4	N29	52% (37-64%)	Fresh lymphocytes Fresh lymphocytes

Advances in Brief

WISH-PC2: A Unique Xenograft Model of Human Prostatic Small Cell Carcinoma[1]

Jehonathan H. Pinthus, Tova Waks, Daniel G. Schindler, Alon Harmelin, Jonathan W. Said, Arie Belldegrun, Jacob Ramon, and Zelig Eshhar[2]

Department of Immunology [J. H. P., T. W., D. G. S., Z. E.] and The Experimental Animal Center [A. H.], The Weizmann Institute of Science, Rehovot 76100, Israel; Department of Urology [J. H. P., J. R.], Sheba Medical Center, Tel-Hashomer 52621 Israel; and Departments of Pathology [J. W. S.] and Urology [A. B.], UCLA School of Medicine, Los Angeles, California 90095

Abstract

Prostatic small cell carcinoma is an aggressive subtype of prostate cancer that usually appears as a progression of the original adenocarcinoma. We describe here the WISH-PC2, a novel neuroendocrine xenograft of small cell carcinoma of the prostate. This xenograft was established from a poorly differentiated prostate adenocarcinoma and is serially transplanted in immune-compromised mice where it grows within the prostate, liver, and bone, inducing osteolytic lesions with foci of osteoblastic activity. It secretes to the mouse Chromogranin A and expresses prostate plasma carcinoma tumor antigen-1, six-transmembrane epithelial antigen of the prostate, and members of the *Erb-B* receptor family. It does not express prostate-specific antigen, prostate stem cell antigen, prostate-specific membrane antigen, and androgen receptor, and it grows independently of androgen. Altogether, WISH-PC2 provides an unlimited source in which to study the involvement of neuroendocrine cells in the progression of prostatic adenocarcinoma and can serve as a novel model for the testing of new therapeutic strategies for prostatic small cell carcinoma.

Introduction

Primary SCCP[3] is a relatively rare form of NE differentiation of PC. Nevertheless, it is clinically important because it is an extremely aggressive tumor with a very poor prognosis. NE differentiation of PC into SCCP is usually identified at the time of progression or recurrence of tumors that were originally classified as conventional adenocarcinoma of the prostate (1). This heterogeneity can be explained by divergent differentiation from multipotent stem cells (2, 3). Upon diagnosis of a small cell component, the clinical course is aggressive with common local and distant failure and a limited median survival duration of 9.8 months (2). It is therefore important to establish an experimental model of this tumor that will enable the testing and exploitation of potential therapeutic modalities.

The optimal treatment for SCCP has not been determined. The tumor often appears mixed with adenocarcinoma of the prostate, and it is usually treated with chemotherapeutic regimens designed for small cell carcinoma of the lung (1). The possible advantage of hormonal agents combined with chemotherapy remains unproven. Indeed, accelerated proliferation of the NE cells may represent an important step in the development of androgen-independent growth of

PC driven by alternative growth signals. di Sant'agnese (4) described the NE cells (also known as the endocrine-paracrine cells of the prostate) as intraepithelial regulatory cells displaying hybrid epithelial, neural, and endocrine characteristics. Although devoid of AR (5), the cells are capable of secreting alternative growth factors such as bombesin, serotonin, somatostatin, calcitonin, and parathyroid hormone-related protein (4–6). The prostatic NE cells express the c-erbB-2 growth factor receptors (7, 8). It was suggested that SCCP is composed of an enriched population of androgen-independent cells whose growth is sustained through alternate paracrine and autocrine pathways (6).

Only one model of human SCCP was reported thus far (9, 10). The WISH-PC2 line described here was derived from a PC patient, and expresses novel PC-specific molecular markers. This model should be extremely useful in studies aimed at the elucidation of critical aspects of the NE differentiation of PC, such as the regulatory mechanisms displayed by the NE cells and the interactions between the disseminated tumor and its various metastatic sites. In addition, the effect of various therapies on the primary tumor and on its disseminated form can be further evaluated.

Materials and Methods

Clinical History. The donor of the WISH-PC2 tumor tissue was a 67-year-old Caucasian male. He was diagnosed with T3N1M1 prostatic adenocarcinoma with a Gleason score of 8 (3 + 5). At the time of diagnosis, his serum PSA level was 53 ng/ml. Hormonal ablation was initiated with s.c. injection of 10.8 mg Goseroline every 12 weeks, resulting in a mild decline in serum PSA levels to a nadir of 40 ng/ml. A year later, while continuing this regimen, the patient complained of obstructive voiding symptoms and serum PSA levels rose to 65 ng/ml. Ultrasonography demonstrated an enlarged obstructing prostate with a significant post-void residual urine volume. Anti-androgen (bicalutamide 50 mg/day) was added to the luteinizing hormone releasing hormone agonist. However, because of worsening of the obstructive voiding symptoms, the patient underwent a palliative transuretheral resection of the prostate. The WISH-PC2 (Weizmann Institute Sheba Hospital Prostate Cancer) xenograft line was established from tissue samples obtained during this operation. The pathological examination revealed poorly differentiated carcinoma infiltrating the smooth muscle, with a typical NE differentiation (Fig. 1). As the patient's disease continued to progress, the hormonal therapy was replaced with chemotherapy (cyclophosphamide, doxorubicin, and vinblastine). However the patient's condition continued to deteriorate, and he expired a few weeks after the initiation of cytotoxic therapy.

Establishment of the Xenograft. Animals and Surgical Procedures. Animals used were 4–10-week-old SCID (c.b-17/Icr Beige or NOD) and nude (BALB/c nu/nu) mice obtained from the pathogen-free animal breeding facilities of the Weizmann Institute of Science. All of the surgical procedures were performed under ketamin + xylazine general anesthesia (127.5 and 4.5 mg/kg, respectively), except for the insertion of s.c. testosterone pellets and s.c. tumor pieces, which was performed under local anesthesia with xylocaine 10% spray (Astra Sweden). The original surgical samples of the tumor were placed on ice, minced into 3–5-mm pieces, and implanted s.c. into SCID mice. After an initial latency period of 4 months, tumor growth was noticed in 30% of the mice. Thereafter the tumor was serially passaged (upon submission of the manu-
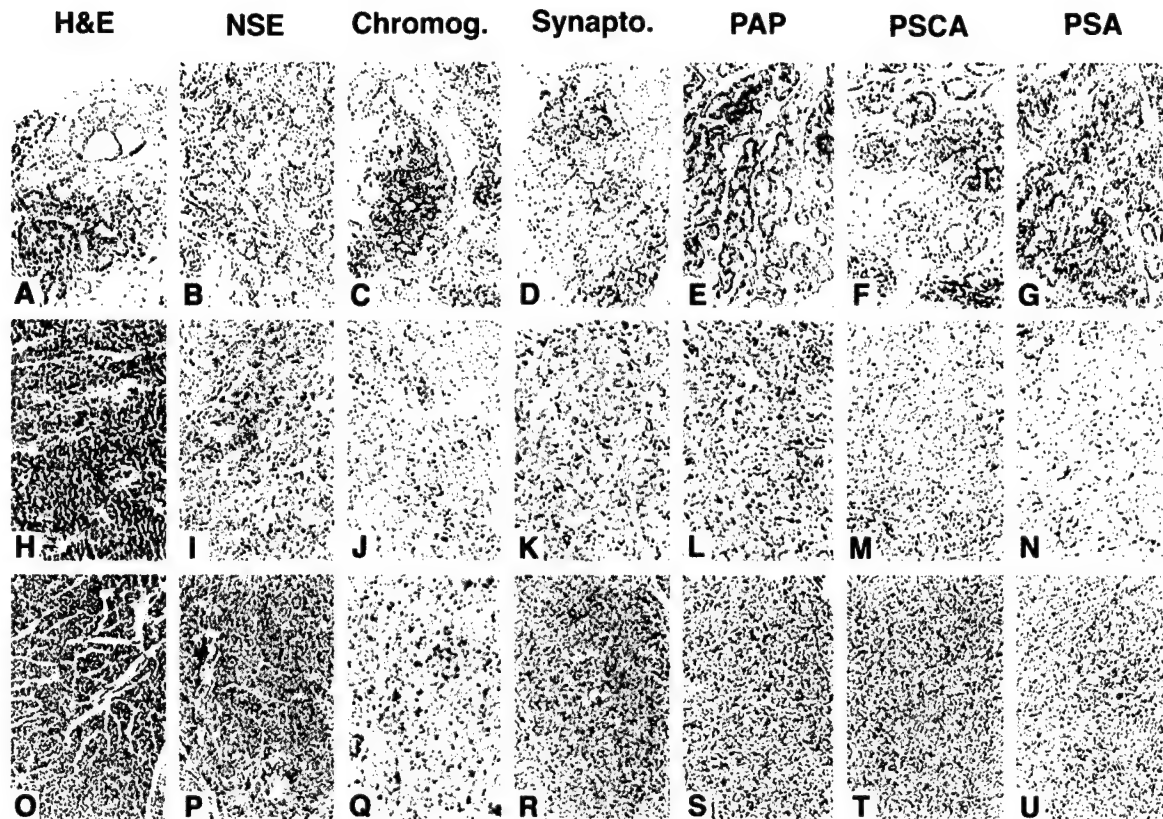
Fig. 1. Immunohistochemical analysis. *A–G*, the patient's prostate biopsy at diagnosis; *H–N*, surgical specimen from which the xenograft originated; *O–U*, the WISH-PC2 xenograft. *Chromog.*, chromogranin A; *Synapto.*, synaptophysin.

script, this line was in its twelfth passage), either s.c. with Matrigel (Becton Dickinson, Bedford MA), as minced tumor pieces, or by direct injection of single-cell suspension into various organs as described below. In some experiments (Fig. 2), a sustained 90-day-release testosterone pellet (12.5 mg/pellet; Innovative Research of America, Sarasota, FL) was implanted s.c. Bilateral transabdominal orchiectomy was performed when stated to achieve androgen ablation.

Tumor size was determined by caliper measurements of length, width, and depth, and the tumor volume (mm³) was approximated using the formula: length × width × depth × 0.5236 (11). Doubling time of the tumor growth was calculated during the logarithmic growth phase of subcutaneously growing tumors.

Injection of Tumors to Various Organs. Orthotopic Injection. Tumor cell suspensions (20 μl) were injected into the dorsal prostatic lobes through a midline lower abdominal incision as described by Stephenson *et al.* (12). A well-localized bleb within the injection site was considered to indicate technically satisfactory injection.

Intraoseos Injection. Through a skin incision in the medial aspect of the hind limb, the femur and tibia were exposed. A hole was made in the cortical bone of the distal femur or proximal tibia with a 27-gauge needle. Tumor cell suspensions (10 μl) were injected through the duct using a 30-gauge needle.

Intrahepatic Injection. A midline abdominal incision was performed to expose the liver. Tumor cell suspensions (10–20 μl) were injected into both hepatic lobes using a 27-gauge needle.

s.c. Injection. Mixtures of tumor cell suspensions in various concentrations with 100 μl of Matrigel basement membrane matrix solution (Becton Dickinson) were injected s.c. using a 27-gauge needle.

Preparation of Single-Cell Suspension. The xenografted tumor tissue was harvested under sterile conditions and placed immediately in cooled HBSS, (Sigma-Aldrich Co., Ltd.). Cells were dissociated under sterile conditions, first by mincing the tissue with scissors to small fragments and then by gentle mechanical homogenization through a stainless still mesh. Viable cells were separated from debris by layering over Ficoll-Paque 400 (Pharmacia Biotech AB, Uppsala, Sweden) and centrifugation at 500 × g for 20 min. Viable cells

at the interface were collected, counted, and resuspended in cooled HBSS to the desired concentrations.

FACS Analysis. Single-cell suspensions made from the xenograft were washed and incubated with primary antibody [anti-CD19, anti-CD20, anti-CD22 (a generous gift from Dr. M. Little, DKFZ, Heidelberg, Germany), PA2.6 anti-HLA-ABC (ATCC HB-118), N29 anti-ErbB-2, and L96 anti-ErbB2, no. 105 anti-ErbB-3 and no. 77 anti-ErbB-4, a generous gift from Prof. Y. Yarden (Ref. 13 and references therein), or an irrelevant U76.6, anti-di-nitrophenol monoclonal antibody]. The samples were then washed and incubated with the secondary FITC-labeled antimouse antibody. Stained cells were resuspended in propidium iodide to identify and exclude dead cells. Analysis was performed on the FACScan flow cytometer (Becton Dickinson).

Immunohistochemistry. Immunohistochemistry was performed on sections from formalin-fixed, paraffin-embedded blocks as described previously (14).

Antibodies used included mouse monoclonal antibodies to PAP, PSA, NSE, chromogranin and synaptophysin, obtained from DAKO Corp., Carpinteria, CA. Antibodies to human AR were purchased from Innovex Biosciences, USA. Antibody to PSCA was a gift from Dr. Robert Reiter (University of California at Los Angeles). After incubation with monoclonal antibodies, slides were incubated sequentially with peroxidase-conjugated rabbit antimouse immunoglobulins, and peroxidase-conjugated swine antirabbit immunoglobulins. Antibody localization was visualized with the diaminobenzidene reaction. Negative controls consisted of substitution of the primary antibody with an isotype matched non-cross-reacting antibody of irrelevant specificity.

Immunohistochemical techniques combined with image analysis were used to detect the presence of bcl-2, P glycoprotein (MDR1), and p53. An experienced commercial laboratory performed these pathological evaluations as well as determination of DNA ploidy and proliferation index of the tumor samples (Quantitative Diagnostic Laboratories).

Serum PSA levels were determined by Immulite Third generation PSA kit (Diagnostic Products Corp., Los Angeles, CA).

Plasma chromogranin-A levels were quantified using an ELISA kit as recommended by the manufacturer (DAKO Denmark).
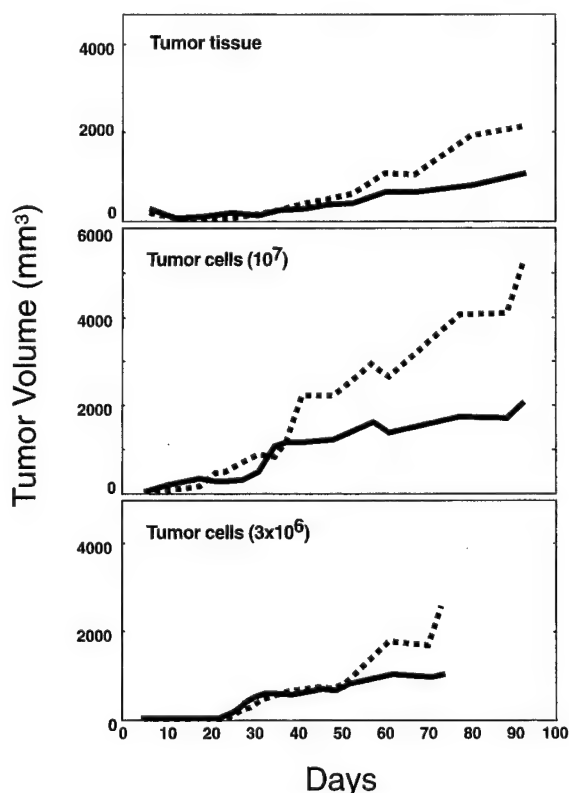
Fig. 2. Effect of androgen on the growth of WISH-PC2 xenograft. The volume of s.c growing WISH-PC2 tumor tissue and dissociated cells grafted s.c. in orchiectomized male SCID mice in the absence (*solid line*) and presence (*dotted line*) of s.c implanted slow-release testosterone pellets.

Western Blot Analysis. WISH-PC2 cell lysate was resolved by 12% SDS-PAGE, transferred to nitrocellulose paper and blotted with polyclonal rabbit anti-PCTA-1/galectin 8 antibodies (a generous gift from Prof. Y. Zick).

RT-PCR. RNA was isolated from WISH-PC2 tissue samples using the TRI Reagent kit (Molecular Research Center, Inc.), and RT-PCR was performed using the Reverse Transcription System (Promega). The quality of all cDNA samples was confirmed by PCR using primers for β-actin. PCR was performed using primers for AR, PCTA-1, PSA, PSMA, PSCA, STEAP, and cytokeratin 8 and 18.

Results and Discussion

NE differentiation of PC is associated with poor prognosis and resistance to anti-androgen therapy (5, 15). NE differentiation can take several forms, including NE SCCP, carcinoid-like tumor or, most commonly, focal NE differentiation in conventional prostatic adenocarcinoma. In this study, we established a xenograft model that could recapitulate many of the clinical characteristics of SCCP. As such, it enables phenotypic characterization and the development and evaluation of possible therapeutic modalities.

Growth Pattern of the WISH-PC2 Xenograft. The patient from whom the WISH-PC2 xenograft was established was initially diagnosed with conventional high-grade adenocarcinoma of the prostate (Fig. 1). The tumor converted to SCCP in parallel to an expeditious clinical course of progression, emphasizing the linkage between the two subtypes of the prostatic tumor (1, 2, 16). Indeed, in the first generation, 20% of the mice into which the tumor pieces were implanted had elevated serum PSA levels. The WISH-PC2 xenograft was established from a tumor-bearing mouse that did not exhibit elevated serum PSA levels. We determined that the WISH-PC2 cells are of human origin and did not result from an overgrowth of the explants by murine cells (17). The xenograft is stained with anti-HLA-A,B,C antibodies (data not shown). In addition, the tumor does

not express the B-cell CD19, CD20, and CD22 differentiation antigens (data not shown), excluding the possibility of being an overgrowth of dormant EBV-transformed human B cells (18). The xenografted carcinoma is highly cognate in its gross histological appearance to the donor's surgical specimen, and it shares the expression of NE tumor markers (Fig. 1): chromogranin A, NSE, and synaptophysin. Notably, chromagranin A is also secreted into the plasma of WISH-PC2-bearing mice, and the plasma concentration of chromogranin A is correlated to the size of the xenograft (data not shown). Hence, chromogranin A can serve as a secreted tumor marker to monitor the growth of this NE SCCP xenograft.

The WISH-PC2 xenograft grows relatively rapidly (Fig. 2) and with a high take rate (visible tumor growth is evident in 90–100% of the animals). Upon s.c. injection of tumor cells or implantation of tumor tissue, growth can be detected within 2–3 weeks. In the presence of Matrigel, the doubling time of the tumor after the s.c. implantation of 3×10^6 cells, is 11 or 13.5 days for a tumor growing in the presence or absence of androgen, respectively, and 15 and 18 days, respectively, for a tumor that arises from s.c. implantation of tissue (70 mg) with and without androgen supplementation. This pattern of androgen-responsive growth most probably reflects an indirect effect of androgen, inasmuch as the tumor cells do not express ARs as expected for SCCP (1, 3). Because the WISH-PCR xenograft has been originated from a mixed-type tumor, there is a possibility that it is a mixture of SCCP with some residual adenocarcinoma cells that are hormone-responsive and expand in the presence of androgen. To test this, we stained the WISH-PC2 tissue that grew for more than 80 days in the presence of a continuous supply of testosterone with anti-PSA and anti-AR antibodies. The results did not reveal any staining above background of these tumors. Moreover, RT-PCR analysis of RNA derived from WISH-PC2 that grew in the absence or presence of androgen was completely negative for PSA and AR (data not shown). Similarly, no PSA could be detected in the sera of mice bearing WISH-PC2, regardless of whether they were hormone-supplemented or not. All these data argue against the possibility that the enhanced growth observed in the presence of androgen (Fig. 2) is attributable to residual androgen-responsive adenocarcinoma cells. Apparently, the serial transfer of WISH-PC2 in the absence of an external source of testosterone in the first few generations provided a selective advantage to the SCCP component over the adenocarcinoma. Androgen was demonstrated to have indirect effects on PC via up-regulation of surrounding stromal vascular endothelial growth factor production and angiogenesis (19). The high growth rate is also reflected by the high proliferation index, depicted by staining with the Ki-67 antibody, and directed at a nuclear antigen expressed in proliferating cells (data not shown).

Growth of WISH-PC2 in Various Organs. To establish a valid model in which to test various therapeutic strategies, we developed a xenograft model using WISH-PC2 cells that would closely model human SCCP and its metastasis. The ability of the WISH-PC2 xenograft to grow orthotopically within the murine prostate (Fig. 3) provides such a model. Interestingly, orthotopically transplanted human small cell lung carcinoma displays a different chemosensitivity pattern compared with the s.c. transplanted model (20).

One of the typical clinical features of SCCP, in contrast to adenocarcinoma of the prostate, is its tendency to develop visceral metastasis. The most frequent metastatic sites of SCCP are the bones (55%), regional and distant lymph nodes (52%), and the liver (48%; Ref. 2). Occasionally liver, lung, and lymph node metastasis can be recovered after s.c. implantation of WISH-PC2. Interestingly, a high incidence of metastasis are found after direct intrahepatic injection of the xenograft cells, especially in surgical wound sites such as that in mice which have undergone bilateral transabdominal orchiectomy for an-
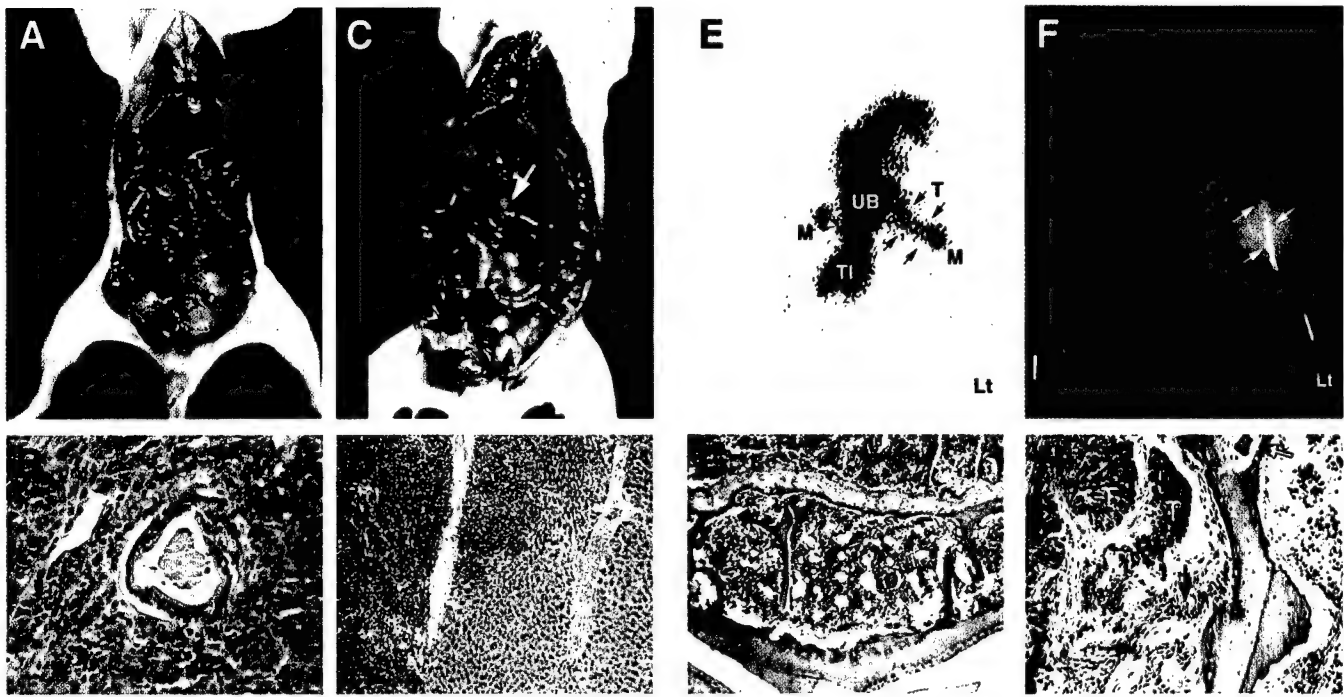
Fig. 3. Growth of WISH-PC2 xenograft in various organs. *A*, orthotopic growth of WISH-PC-2 within the murine prostate, forming a large pelvic tumor mass (41 days after the injection of 2.5 × 10^{6} tumor cells into the prostate). *B*, the tumor cells infiltrate the murine prostate microscopically, distracting its normal architecture. (H&E staining; original magnification ×400). *C*, WISH-PC-2 tumor growth within the murine's left hepatic lobe (*white arrow*) 58 days after an intrahepatic injection of 5 × 10^{6} tumor cells. *Black arrows*, multiple metastasis, including at surgical wound site of bilateral orchiectomy performed simultaneously with the direct intrahepatic injection. *D*, histological view of WISH-PC2 tumor nodule within the murine liver. Note the zone of compressed hepatocytes adjacent to the tumor and the high mitotic figure of the tumor cells. Also of note is the NE appearance of the WISH-PC2 tumor composed of clusters and nests of tumor cells interlaced by a delicate reticular connective tissue. (H&E staining; original magnification ×100). *E*, Tc-99m-MDP bone scan of a mouse 87 days after the injection of 1.5 × 10^{6} WISH-PC2 cells to the left proximal tibia and PBS to the right one. An increased uptake of the radioisotope indicates osteoblastic activity restricted to the site of tumor injection (*arrows*) and not in the contralateral control site (*TI*, site of the tail injection of the radioisotope; *UB*, urinary bladder; *M*, marker indicating the location of the right and left legs; *T*, tumor.). *F*, plain radiography to the same mouse, demonstrating lytic distraction of the bone only at the site of tumor injection (*white arrows*) and not in the contralateral tibia. *G*, histological view of the right proximal tibia of the mouse that was injected with PBS demonstrating normal bone architecture, including epiphysis, bone marrow cells, bone trabeculi. (H&E staining; original magnification ×100). *H*, bone architecture in the left proximal tibia of the mouse into which the WISH-PC2 cells were injected is distorted by tumor cells (*T*). Note a bony "plaque" surrounded by abundant osteogenitor cells and osteoblasts (*black arrows*). On the right upper side of the bony plaque, lytic changes are formed by tumor cells (*open arrow*; H&E staining; original magnification ×200).

drogen ablation. (Fig. 3). This may represent preferential seeding of the circulating tumor cells in granulating tissue, which is rich in neovascularization and local growth factors. The WISH-PC2 xenograft cells can also grow in the host bones, inducing new bone formation mixed with lytic distraction of the bone, a typical feature of SCCP bone metastasis (21). This is demonstrated both radioisotopically and radiographically (Fig. 3). In contrast with WISH-PC2, the SCCP xenograft UCRU-PR-2, reported previously, failed to grow in the liver (10), and no data are available on the ability of UCRU-PR-2 to grow orthotopically within the prostate or bone.

Malignant Phenotype of the WISH-PC2 Cells. Next we tested WISH-PC2 cells for the expression of several prostate specific markers. Table 1 lists various molecular markers whose expression was evaluated on WISH-PC2. The xenograft does not express PSA, PSMA, PSCA, or PAP. Nevertheless, its prostatic origin is supported by the following molecular markers: (*a*) expression of cytokeratin 8 and 18 common to PC and prostatic secretory cells (3); (*b*) expression of PCTA-1, which is a surface marker of PC and its precursor, prostatic intraepithelial neoplasia, but is not found on normal prostate or benign prostatic hyperplasia (22); and (*c*) expression of the STEAP. This recently described surface marker (23) is highly expressed at all stages of PC and does not seem to be modulated by androgen. Although STEAP is also expressed in multiple cancer cell lines, its expression in normal human tissues is restricted to the prostate and bladder.

Immunostaining of WISH-PC2 xenograft sections demonstrated that the tumor expresses adverse pathological features reflecting the aggressive nature of this tumor. These include aneuploid DNA con-

tent, bcl-2 protein, and mutated p53 (Table 1). Although previously prostate NE cells were reported not to express the antiapoptotic bcl-2 marker (24), these cells were terminally differentiated, nonproliferating cells. The WISH-PC2 cells represent malignant prostatic NE cells.

Table 1 *Phenotypic features of WISH-PC2*

Feature	Expression
DNA ploidy	Aneuploid
Proliferative Index (Ki-67)[a]	High
Bcl-2[a]	Positive
Mutated p53[a]	Positive
MDR1 gene product[a]	Negative
PSA[a,b]	Negative
PSCA[a,b]	Negative
PSMA[b]	Negative
PAP[a]	Negative
AR[a,b]	Negative
STEAP[b]	Positive
PCTA-1/galactin-8[b,c]	Positive
Cytokeratin 8[b]	Positive
Cytokeratin 18[b]	Positive
Chromogranin A[a,d]	Positive
NSE[a]	Positive
Synaptophysin[a]	Positive
Her-2/neu[e]	Positive
Her-3/neu[e]	Positive
Her-4/neu[e]	Positive
MHC class-I[e]	Positive

[a] Determined by immunohistochemistry.
[b] Determined by RT-PCR.
[c] Determined by Western blot analysis
[d] Determined by ELISA of murine host plasma.
[e] Determined by FACS analysis.

It is therefore intriguing to speculate that both bcl-2 and defective p53 allow these AR-negative cells to survive and overtake the adenocarcinoma cells, which undergo apoptosis in response to androgen deprivation and, potentially, chemotherapy.

FACS analysis using monoclonal antibodies against the epidermal growth factor receptor *erb-B* family revealed that WISH-PC2 expresses erb-B2, erb-B3, and erb-B4 on its surface. The expression of these growth factor receptors was stable through all of the passages of the tumor (data not shown). Iwamura *et al.* (7) demonstrated immunostaining of c-erb-B2 on prostatic NE cells using polyclonal antibodies. To the best of our knowledge, no data have been reported to date concerning the coexpression of erb-B3 and/or erb-B4 with erb-B2 on prostatic NE cells, a combination that is necessary for the binding of Neuregulin ligands to these receptors and for their activation (13). The presence of the erb-B set of receptors may provide an alternative pathway of growth signaling for the androgen-independent proliferation of these cells, either directly or by regulating NE peptides that function in an autocrine or paracrine manner.

Potential Application of the WISH-PC2 in Therapeutic Models. Prostatic small cell carcinoma is a notoriously aggressive malignancy with a very poor prognosis (21). No effective treatment for SCCP has been established, most probably because of the limited patient population and the aggressiveness of the disease. WISH-PC2 provides an *in vivo* model for the evaluation of different possible therapeutic strategies for SCCP with an inherent plasma tumor marker (chromogranin A).

The issue of whether castration is a justified treatment for SCCP is still unresolved. The progression from adenocarcinoma of the prostate to SCCP usually appears after castration (1). However, based on the evidence that most SCCP are mixed with adenocarcinoma of the prostate, the common practice is to combine hormonal and cytotoxic therapy (21, 25). The fact that androgen supplements somewhat increase WISH-PC2 tumor growth (Fig. 2) suggests that androgens may enhance the growth of the AR-negative xenograft, probably via an indirect effect on the surrounding stroma (19). It is therefore possible that in the case of SCCP, especially in those present as mixed histology (adenocarcinoma and small cell elements), hormonal manipulation may slow tumor progression.

The WISH-PC2 model can serve as a useful model for testing established and novel cytotoxic drugs. Targeted drug delivery to the various anatomical sites of visceral distribution of SCCP, such as liver or bones, may be readily tested in this model system. The WISH-PC2 xenograft, lacking the p-170 multi-drug efflux pump (MDR1; Table 1), that mediates the MDR phenotype, is therefore susceptible to chemotherapy. It has still to be demonstrated however, whether the WISH-PC2, expressing mutated p53 and bcl-2 are susceptible to apoptosis-inducing drugs.

In conclusion, the WISH-PC2 SCCP xenograft is an excellent source for NE prostatic cells and their factors in studies of intercellular interactions that take place during PC progression. Moreover, this novel human xenograft can serve as a model for the exploitation of new therapeutic modalities on this aggressive variant of PC.

Acknowledgments

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Review article

Functional expression of chimeric receptor genes in human T cells

Zelig Eshhar*, Tova Waks, Alain Bendavid, Daniel G. Schindler

Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Abstract

Tumor immunotherapy has been limited to date by the poor antigenicity of most tumors, the immunocompromised state of many cancer patients, and the slow tumor penetration and short half-life of exogenously-introduced anti-tumor antibodies. Our group has developed a model immunotherapy system using a chimeric construct containing an antibody V region fused to a T cell activation molecule (T body) introduced by transfection into cytotoxic T cell lines, or populations of activated primary T or natural killer (NK) cells. In this study we have optimized the conditions needed for efficient transduction of human peripheral lymphocytes (PBL) using retroviral vectors pseudotyped with the gibbon ape leukemia virus (GaLV) envelope. Selection of packaging cells producing high virus titers was performed following transfection with constructs containing the green fluorescent protein (GFP), and FACS sorting. As a model chimeric receptor gene we used a tripartite construct consisting of a single-chain anti-TNP antibody variable region linked to part of the extracellular domain and the membrane spanning regions of the CD28 coreceptor molecule and joined at its 5' end to a gene fragment encoding the intracellular moiety of the γ activation molecule common to the Fcϵ and Fcγ receptors. Enriched preparations of retrovectors containing this chimeric receptor and the GFP gene could stably and efficiently transduce human PBL co-activated by anti-CD3 and anti-CD28 antibodies. In routine experiments, the transgene was expressed in 35–70% of the human T cells. Such lymphocytes express the chimeric receptors on their surface and upon stimulation with hapten immobilized on plastic they can produce IL-2. Transfectomas activated in this manner also undergo specific proliferation in the absence of exogenous IL-2. Moreover, the transduced lymphocytes could effectively lyse target cells expressing the TNP hapten on their surface. These studies establish the conditions for the optimal transfection of effector lymphocytes to redirect them against a variety of tumor targets. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Chimeric receptor; Retrovectors; T cells; Gene expression; Gene delivery

1. Introduction

To overcome the inability of antibodies to eliminate solid tumors on the one hand as well as the lack of specific of anti-tumor T cells in many malignancies our group has pioneered the T body approach (Gross et al., 1989; Eshhar et al., 1993). In the T-body methodology, we have joined the two approaches of adoptive T cell immunotherapy and

Abbreviations: GFP, green fluorescent protein; scFv, single chain Fv; MuLV, Moloney murine leukemia virus; GaLV, gibbon ape leukemia virus; TNP, trinitrophenol; PBL, peripheral blood lymphocytes; NK, natural killer cells

*Corresponding author. Tel.: +972-8-934-3965; fax: +972-8-947-4030.

E-mail address: zelig.eshhar@weizmann.ac.il (Z. Eshhar).

antibody therapy to genetically engineer an improved 'immunocytolysin', an antibody V region attached to a cytotoxic T cell signaling molecule and responsible for recognition and activation. Genes for chimeric T cell receptors have been constructed containing the coding sequence of an antibody-derived single-chain Fv directed against a tumor associated antigen, attached to the transmembrane and intracytoplasmic sequences of T cell signaling molecules. These genes are then transfected into cytotoxic T cells, thereby conferring upon them the ability to specifically recognize and kill tumor cells. The scFvR design we have developed combines antibody recognition and T cell signaling in one continuous protein and has been used to endow murine and human effector cells with non-MHC-restricted, antibody-derived specificity. This T-body approach combines the advantage of antibody specificity with the homing, tissue penetration, and target cell destruction mediated by T lymphocytes.

Using this technology, in the past 10 years we have been able to redirect lymphocytes to predefined targets and to endow them with antibody-like specificity. We have designed and developed several receptor configurations, identified the optimal receptor molecules to give a strong effector response, and have chosen a single chain antibody configuration that yields effective antigen binding for virtually all antibodies (Eshhar et al., 1995; Fitzer-Attas et al., 1998). We have transfected effector cells including the mouse MD45 cytotoxic cell line, human NK (Bach and Eshhar, 1995) and rat basophilic cell lines (Bach et al., 1994) and human tumor infiltrating lymphocyte populations.

The use of redirected effector cells has potential in the immunotherapy of virtually any type of cancer in which tumor antigens, not present on normal tissues, are expressed and shared between patients. Using in vivo model systems, lymphocytes transfected with a chimeric receptor of anti-folate binding protein specificity developed in our lab, were shown to eliminate experimental tumors in mice (Hwu et al., 1993, 1995). In addition, we have made a receptor construct containing anti-HER2 antibodies, which is able to mediate cytolysis when transfected into cytolytic mouse hybridoma cells.

For clinical application, patient-derived lymphocytes will be transfected with a chimeric receptor gene encoding an scFv specific to an antigen expressed by the tumor. After expansion in vitro, such genetically engineered cells will be reinfused into the patient where they are expected to undergo activation at the tumor site and reject the tumor, either by direct cytotoxcicity and/or by causing a local inflammatory response. One of the major technical limitations to this scenario is inefficient gene transfer into T cells. For stable gene expression, the only vectors approved for use in humans are adeno-associated and certain retroviral based vectors. While adeno-associated viruses (AAV) fail to infect T cells, some promising results have been obtained using Moloney murine leukemia virus (MuLV)-based vectors for gene delivery into T cells (Mavilio et al., 1994; Bunnell et al., 1995). It was demonstrated that transduction of human T cells can be augmented by pseudotyping the MuLV with the envelope protein (env) of the Gibbon ape leukemia virus (GaLV) (Bunnell et al., 1995; Lam et al., 1966). For integration into the recipient cell genome, retroviral-mediated gene transfer requires dividing cells. A significant enhancement of gene delivery into human T cells has been recently obtained by combining T cell activation using anti-CD3 plus anti-CD28, and transduction in the presence of fibronectin derived peptide (Pollok et al., 1998, 1999; Dardalhon et al., 2000).

Here we increased the efficacy of functional expression of chimeric receptor genes in primary human T lymphocytes by a procedure that combines optimal T cell activation with improved retrovectors. Such a procedure can be readily applied to the immunotherapy of human cancer using ex vivo transduction of patient lymphocytes with chimeric receptors derived from antibodies specific to surface antigens expressed by the tumor in question.

2. Materials and methods

2.1. Retroviral packaging cell lines

The packaging cell lines used include the ecotropic GP+E-86, the amphotropic PA317 (obtained from ATCC), and PG13, which expresses the GaLV env (Miller et al., 1991). 293T cells were a generous gift from R.A. Willemsen (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). All cells were

cultured in DMEM medium (GIBCO-BRL) supplemented with 10% FCS (GIBO-BRL, Paisley, UK), L-glutamine solution (2 mM), sodium pyruvate 1 mM, 100 Units/ml penicillin and 100 µg/ml streptomycin (Biological Industries, Israel).

2.2. Antibodies and reagents

Anti-human CD3 antibody was purified from the OKT3 hybridoma cell (ATCC) culture supernatant. Anti-CD28.2 was obtained from PharMingen, (San Diego, CA). SP6, an anti-TNP monoclonal antibody (mAb), and 20.5, an anti-Sp6 idiotype mAb, were both provided by G. Kohler (Max-Planck Institute for Immunology) (Rusconi and Kohler, 1985). Fluorescein-labeled anti-mouse Ig antibodies were purchased from Jackson Immuno Research Laboratories Inc (West Grove, PA).

2.3. Transfection/infection

2.3.1. 'Ping-pong' method

First, 3×10^5 GP+E-86 cells and 2×10^5 PA317 cells were co-cultured in a 100-mm plate (NUNC, Roskilde, Denmark). A day later, medium was changed and transfection was performed by adding 20 µg of plasmid DNA in $CaPO_4$ (Mammalian Transfection Kit, Stratagene, La Jolla, CA). After 24 h, the plate was washed with PBS and supplemented with fresh medium. When the culture reached near confluence (24–48 h post transfection), viral supernatant was collected for infection. For infection, 2×10^5 PG13 or 293T cells were plated in a 100-mm plate; the next day, the medium was replaced with 5 ml of viral supernatants in the presence of 4 µg/ml of Polybrene (Sigma) at 37°C, 7.5% CO_2 for 7 h. After 48 h, the infection efficiency was evaluated by monitoring GFP expression (see below). All vector-containing retroviral supernatants described in this study were harvested after a 24-h incubation of near-confluent packaging cells grown in 5 ml fresh medium in a humidified incubator at 32°C, 7.5% CO_2

2.4. Activation of lymphocytes

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by centrifugation through Ficoll Paque Plus (Pharmacia Biotech, Uppsala, Sweden), and cultured in RPMI 1640 medium (GIBO-BRL) supplemented with 10% FCS, L-glutamine solution 2 mM, 100 Units/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol.

Cells (10^6/ml in a well from a 24-well plate) were stimulated for 2 days on Falcon non-tissue culture-treated 24-well plates pre-coated with anti-CD3 plus anti-CD28 antibodies. Immobilization of the antibodies was performed by adding 0.5 ml/well of antibody (1 µg/ml PBS of each of the antibodies/well) to the wells overnight at 4°C; the plates were washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2.5. Lymphocyte transduction

Activated lymphocytes were harvested from the stimulating plate, washed and plated on a RetroNectin™ (FN) (Takara Shuzo Ltd. Otsu, Japan) coated plate at 0.5×10^6/well with 2.8 ml of viral supernatant supplemented with 50 U/ml of IL-2 (recombinant human IL-2 Chiron, Amsterdam, The Netherlands). After 4–6 h at 37°C, 7.5% CO_2, the viral supernatant was gently removed and replaced with RPMI–FCS+50U/mlIL-2 and cells were incubated overnight at 37°C, 5% CO_2. The same transduction process was repeated on the next day. On the day following the second infection, lymphocytes were harvested by vigorous flushing and washing of the wells. The cells were re-suspended in RPMI–FCS medium with 150 U/ml of IL-2, and incubated in 37°C, 5% CO_2. FN-coated plates were prepared by incubating non-tissue culture-treated 24-well plates (Falcon) with 1 ml/well of FN (12 µg/ml) overnight at 4°C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2.6. Flow cytometry

Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Green fluorescent protein (GFP) was measured on FL1 (488 nm excitation and 530 nm emission filter). Viability was determined by assessing propidium iodide (PI) (Sigma) staining using FL2 at 488 nm excitation and 585 nm emission. Expression of scFv (Sp6) on the surface of the cells was evaluated by

immunofluorescent staining using anti-Sp6 mAb (20.6) and rhodamine–phycoerythrin (PE) labeled anti-mouse Fab′ antibody, measured on FL2. Cells were sorted on a FACSsort Plus (Becton Dickinson) according to GFP fluorescence. Cloning of packaging cells from single cell/well positive cells sorted directly into 96-well plates.

2.7. Cell proliferation and cytotoxicity assays

The 96-well plates (non tissue culture treated, NUNC) were coated overnight with 2 µg/well of antigen (TNP-FγG) at 4°C, washed with PBS and blocked with 1% BSA on the following day. Alternatively, for stimulation, various targets were irradiated (12 000 R) and TNP-modified as described previously (Gross et al., 1989). Transduced lymphocytes were washed twice and resuspended in culture medium RPMI–FCS with 50 µM 2-mercaptoethanol. Cells were left for 2–4 h in the incubator to eliminate any remaining IL-2. Transduced lymphocytes (1×10^5/well) were plated on antigen (TNP-FγG), coated plates or cocultured with TNP-modified target cells. At different time points, 50 µl of the cell culture was removed to be tested for cell proliferation by the MTT assay (Mosmann, 1983).

Evaluation of the cytotoxicity of transduced lymphocytes was performed by the ^{51}Cr release assay as described before (Gross et al., 1989).

3. Results and discussion

3.1. Vector and chimeric gene constructs

In order to establish the optimal conditions for transduction of primary human T cells, we tried several systems reported to be efficient for gene delivery of chimeric receptor genes. The BULLET system described by Weijtens et al. (1998) was attempted first; we then evaluated the STITCH vector, a modification of the BULLET system which was developed by the same group (Willemsen et al., 2000). This system involves the transient transfection of three plasmids to produce a transducing retrovirus. One plasmid codes for the envelope protein, which could be from an ecotropic virus, an amphotropic virus, or from the gibbon ape leukemia virus (GaLV). Another plasmid supplies the gag and pol proteins. The transfer vector with the LTRs and packaging signal carries an insert with the gene of interest (Fig. 1). In our experiments, this insert was specially designed. The scFv used in our initial experiments was from an antibody with specificity to trinitrophenol (TNP). This facilitates the testing of the genetically modified lymphocytes for their ability to kill a wide variety of cells as any cell can be made a target simply by treatment with picryl chloride to label the cell surface with the TNP group. Another advantage of this scFv is that we have available an anti-idiotypic monoclonal antibody for detecting by FACS expression of this scFv on the cell surface. In the construct used, the co-stimulatory molecule CD28 is genetically fused to this scFv. This provides spacing of the scFv from the cell surface and permits the formation of heterodimers with endogenous CD28 molecules. The CD28 molecule has previously been used in chimeric receptor constructs (Vallina and Hawkins, 1996; Finney et al., 1998). In addition, CD28 signaling has been shown to prevent apoptosis of the lymphocytes. Since CD28 is homologous to CTLA4 and since the structure of CTLA4 has been determined we have employed the latter as a model for the structure of CD28. Alignment of their amino
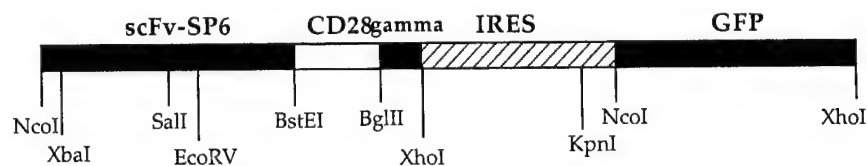

Fig. 1. Structure of the chimeric receptor-GFP gene used in this study. Sp6-scFv is a single chain Fv containing the sequence of the leader, V_L, linker and V_H of the anti-TNP Sp6 antibody, CD28 sequence contains the CD28 hinge region, transmembrane region, and cytoplasmic region. The γ chain sequence contains the cytoplasmic region (starting from the QVR at a *BglII* site). This deletes CRLKI at the beginning of the cytoplasmic region of the Fcε receptor γ and contains the signaling portion of the receptor. IRES is an internal ribosomal entry site (IRES) that permits expression of the GFP from the same transcript as the chimeric receptor.

acid sequences to that of a mouse heavy chain reveals that the sequence in CD28 IHV matches the *Bst*E II site sequence in our single chain VTV (Holm and Sander, 1996). Therefore, the junction was made so that the junction is at a valine which is conserved between the end of the framework 4 beta sheet of the variable region of the antibody (Kabat numbering 111) and a valine which is just after the last beta sheet in CTLA4.

Human CD28 was cloned from PBLs and Jurkat cells using the following primers:

#7966 *Bst*E II primer for homodimer CD28
5′ CCGGTCACCGTGAAAGGGAAACACCTTT-GTCC
#7967 reverse 3′ primer
5′ CGCTCGAGGTGTCAAGATCTATAGGCTG-CGAAGTCGCGTGG

In this way the single chain is attached to the CD28 hinge region, transmembrane region, and cytoplasmic region.

The γ chain cytoplasmic region (starting from the QVR at a *Bgl*II site) was next spliced to the end of the CD28 sequence. This deletes CRLKI at the end of the transmembrane or the beginning of the cytoplasmic region of the Fcε receptor γ chain (Spencer et al., 1993) (Fig. 1). This contains the signaling portion of the receptor and completes the gene for the chimeric T cell receptor.

To enable better tracking of retroviral expression, the gene for green fluorescent protein (GFP) was placed downstream of the chimeric receptor gene separated by an internal ribosomal entry site (IRES) to permit expression of the GFP from the same transcript as the chimeric receptor. Retroviruses with a single transcript containing a desired gene and GFP driven by an IRES have been made previously (Aran et al., 1998; Levenson et al., 1998). In our case, the GFP gene was placed in the retroviral vector pSAM-EN (Morgan et al., 1992) in place of the drug resistance gene, and the chimeric receptor gene was inserted into the cloning site before the IRES. Lack of a second promoter avoids the problem of promoter interference. It also enables linked expression so that GFP fluorescence may be used to assess expression of the scFv. Transduced cells can be observed visually by fluorescence microscopy, thereby permit-

ting the tracking of transduced cells in vivo (Persons et al., 1997; Bagley et al., 1998).

3.2. Establishment of stable vector producing cell lines

Retrovirus-containing supernatants prepared from the 293T cells as described above gave up to 15% transduction of fresh 293T cells as estimated by the proportion of cells exhibiting GFP fluorescence (data not shown). The percentage and level of gene expression in this transient system varied and depended on the transfection efficiency and culture conditions. To obtain a more reproducible system, we have attempted to establish a stable vector-producing cell line by selection for high titer retrovector producing clones. Fig. 2 schematically describes the protocol we have employed for these studies. Briefly, the chimeric receptor gene incorporated in a BULLET or STITCH vector was transfected into the amphotropic PA317 and ecotropic GP+E-86 lines, which were co-cultured in order to increase the viral titer. The supernatant obtained was used to infect a series of packaging cell lines whose supernatants, containing the pseudotyped retrovector, were titered on 293T cells and in parallel tested for their ability to infect activated human PBL (as described in Materials and methods). In several experiments,
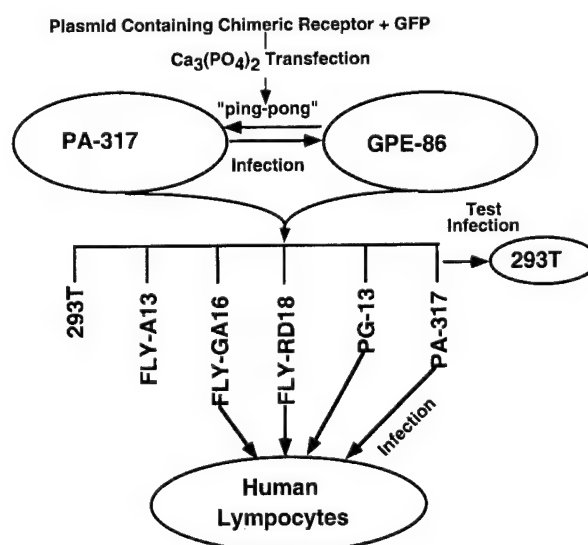

Fig. 2. A diagram of the protocol used for the generation and selection of a high titer packaging cell lines.

the PG13 containing the GaLV envelope, was highly infected, produced the highest titer of virus and resulted in the best gene transfer into the human T cells (data not shown). It was previously shown that the receptor to the GaLV envelope, GLVR-1 is increased following T cell stimulation (Lam et al., 1966). The heterogeneous population of packaging cells obtained was sorted for GFP expression by FACS and the sorted population was further sub-cloned (Fig. 3). As can be seen in the figure, virtually all the cells expressed GFP following a single cycle of sorting.

3.3. Infection of primary human T cells

For optimal transduction of human peripheral blood lymphocytes with retroviruses, the lymphocytes must be activated. In our protocol, they are grown in RPMI with fetal calf serum without IL-2 with plastic bound anti-CD3 and anti-CD28 antibodies for 48 h. They are then infected with supernatant containing virus from the packaging cell line on plates coated with recombinant fibronectin fragments (Retronectin™). The infection is performed in the presence of IL-2 for 5–7 h, twice over 48 h as described in Materials and methods. Low concentrations of IL-2 were used to prevent the propagation of natural killer cells. Higher concentrations are used for the production of lymphokine-activated killer (LAK) cells (Jadus et al., 1988). RetroNectin™ is a recombinant fibronectin fragment which contains the connecting segment, cell binding domain and heparin binding domain (Hanenberg et al., 1996). Its significantly enhances the infection efficacy, since both the retroviral particles and T cells bind to it (T cells through the VLA integrin, the expression of which is increased following anti-CD3/CD28 stimulation).

Several days following transfection, the cells were phenotyped for surface markers and for expression of the chimeric gene using anti-idiotypic antibody and evaluated for expression of GFP. The percentage of transfected cells, 3–4 days following transduction was in the range of 35–70% and remained stable for about 10–14 days. After this period, the proportion of GFP positive cells began to slowly drop unless they were re-stimulated by anti-CD3/CD28, PHA or allogeneic cells. Phenotypically, the transfected cell population consisted of about 30% CD4, 70% CD8

and 15% CD56 positive cells. The population of NK cells (CD56+) dropped during culture in the low IL-2 concentration.

Since throughout all the selection and sorting procedures we followed GFP expression, and since the chimeric receptor gene and GFP were in two separate cistrons, it was important to check whether the expression of GFP truly indicated chimeric receptor expression. Fig. 4 shows the FACS pattern of transduced T cells that were stained with anti-idiotypic antibody against the Sp6 anti-TNP scFv and also analyzed for GFP expression.. It is clear that most of the GFP expressing cells also expressed the chimeric receptor on their surface.

3.4. Functional expression of the chimeric receptor genes in human T cells

In vivo, effector lymphocytes redirected with chimeric receptors are expected to reach their destination, undergo specific stimulation at the target site to either kill the target or secrete cytokines that in turn will cause inflammation and result in elimination of the tumor. Ideally, the effector lymphocytes may differentiate into memory cells which will stay on-guard and prevent reappearance of the tumor in question. To check the ability of the chimeric-receptor expressing human T cells to perform these functions in vitro, we tested their ability to undergo proliferation and to produce cytokines in response to specific antigen as well as their ability to kill specific target cells.

Fig. 5 depicts the results of an experiment in which T cells expressing the SP6-scFv-CD28-gamma chimeric receptor (anti-TNP specific) were triggered for specific proliferation following stimulation on antigen (TNP-FγG) coated culture-wells and the removal of exogenously supplied IL-2. While lymphocytes did not proliferate following 3 days of culture in the absence of IL-2, in the presence of TNP-FγG (hapten/protein ratio >2) they propagated quite well for at least 1 week. In the presence of antigen excess (TNP$_{58}$-FγG) cells started to die following 7 days, most likely because of overstimulation or antigen-induced cell death (AICD) that was not prevented by the presence of CD28 in the signaling receptor. Notably, the cell proliferation, and IL-2 production (data not shown), was triggered
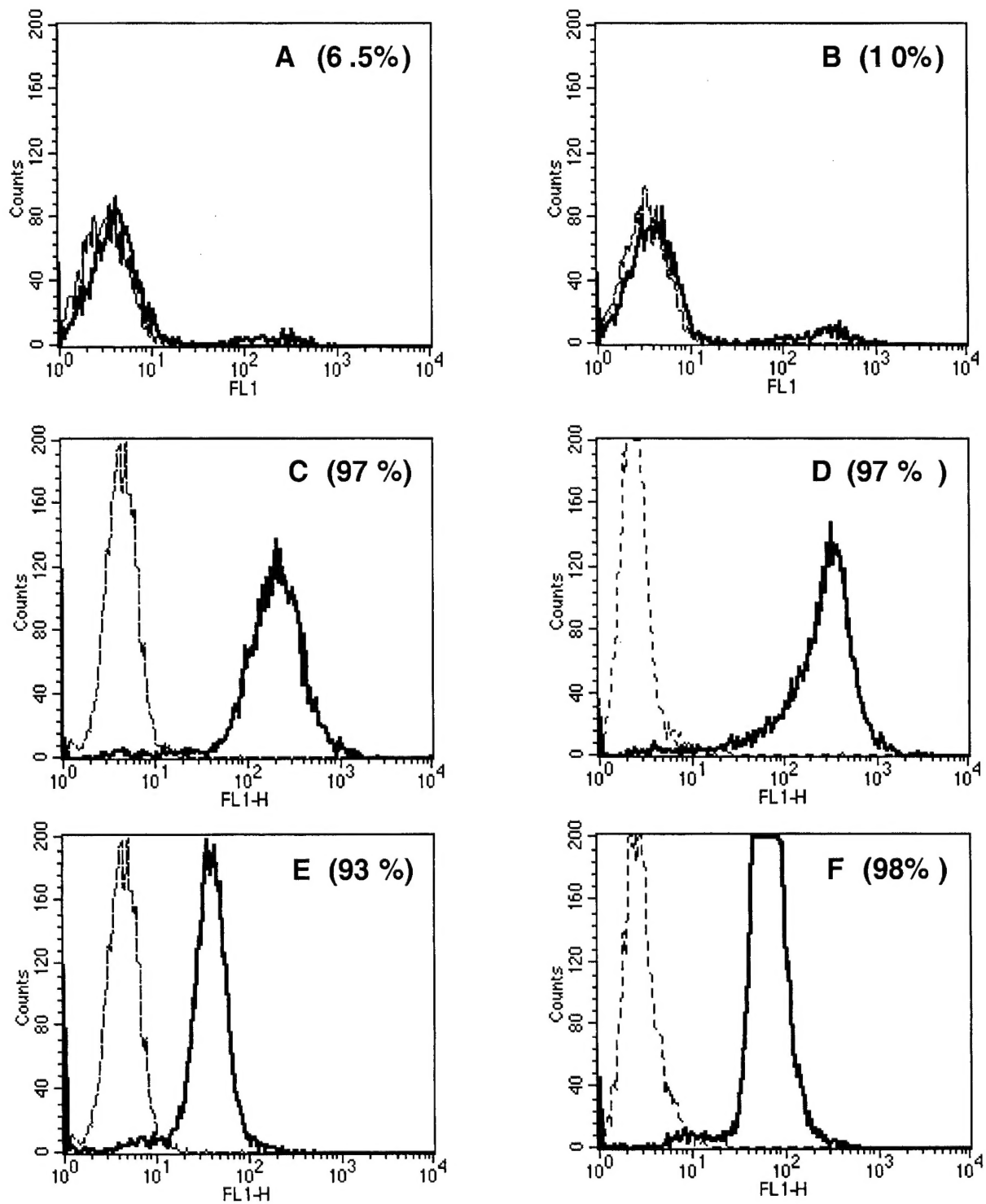
Fig. 3. GFP fluorescent pattern of two packaging cells at different stages of their production. A, C, E represent cell lines derived from the amphotropic PA317 cell; B, D, F represent cell lines derived from the PG13 cell containing the GaLV envelope. A, B-packaging cells 24 h after their transduction by cell-free supernatants obtained from the 'Ping-Pong' step (Fig. 2). C, D represent the GFP expression in bulk-sorted packaging cells. E, F represent clones selected for high vector titer from the bulk-sorted population.
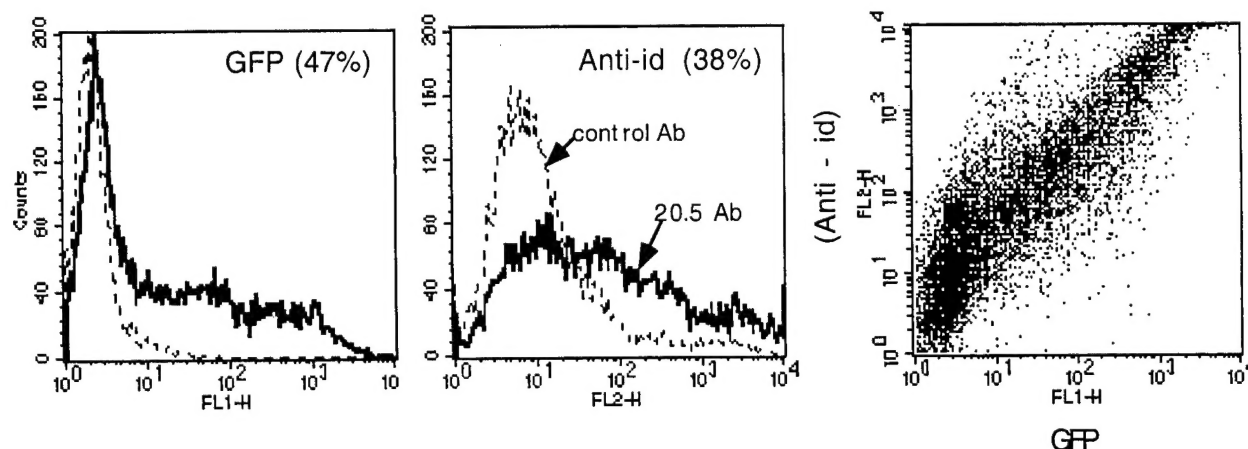
Fig. 4. Co-expression of the chimeric receptors and GFP transduced human T cells. Fluorescent pattern of GFP expression (FL1) and surface expression of the chimeric receptor stained with biotin–anti-idiotypic antibody (20.5) and avidin–phycoerythrin.

by the chimeric receptor in the absence of any other stimulatory and co-stimulatory signal, showing that both signal I and II can be induced by our tripartite receptor. The T cells bearing the chimeric receptors could also specifically and efficiently kill various TNP-modified target cells regardless of their species or tissue origin (Fig. 6). The genetically engineered lymphocytes were able to efficiently cytolyse their targets following 3 weeks culture in the presence of IL-2, even in the absence of preactivation before the

cytotoxicity assay. At this stage, the level of non-specific LAK-mediated killing was low.

Taken together, the results described above demonstrate that under the conditions used, PBL-derived T cells can be efficiently transduced to express high levels of chimeric receptors. The chimeric receptor could induce the T cells to specifically proliferate and produce cytokines as a result of specific stimulation by antigen alone without additional stimulation. The genetically engineered cells maintain their abili-
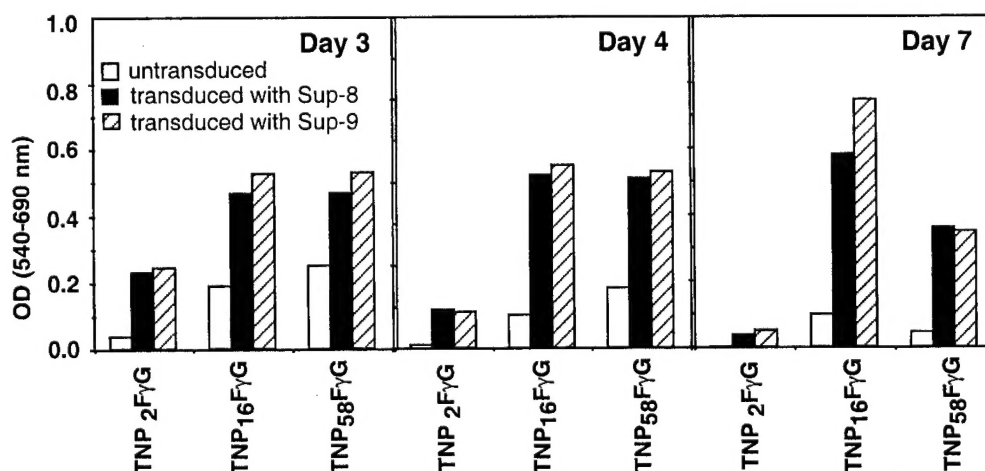

Fig. 5. Proliferation of chimeric receptor expressing lymphocytes stimulated with antigen. T lymphocytes transduced with retrovector preparations made of two packaging clones 8 and 9, as well as non-transduced cells were cultured in the absence of IL-2 on plastic-immobilized antigens containing different hapten groups (TNP) per $F\gamma G$ carrier. Cell proliferation at different days in culture was monitored by the MTT colorimetric assay. Note that the culture medium was changed at day 4.
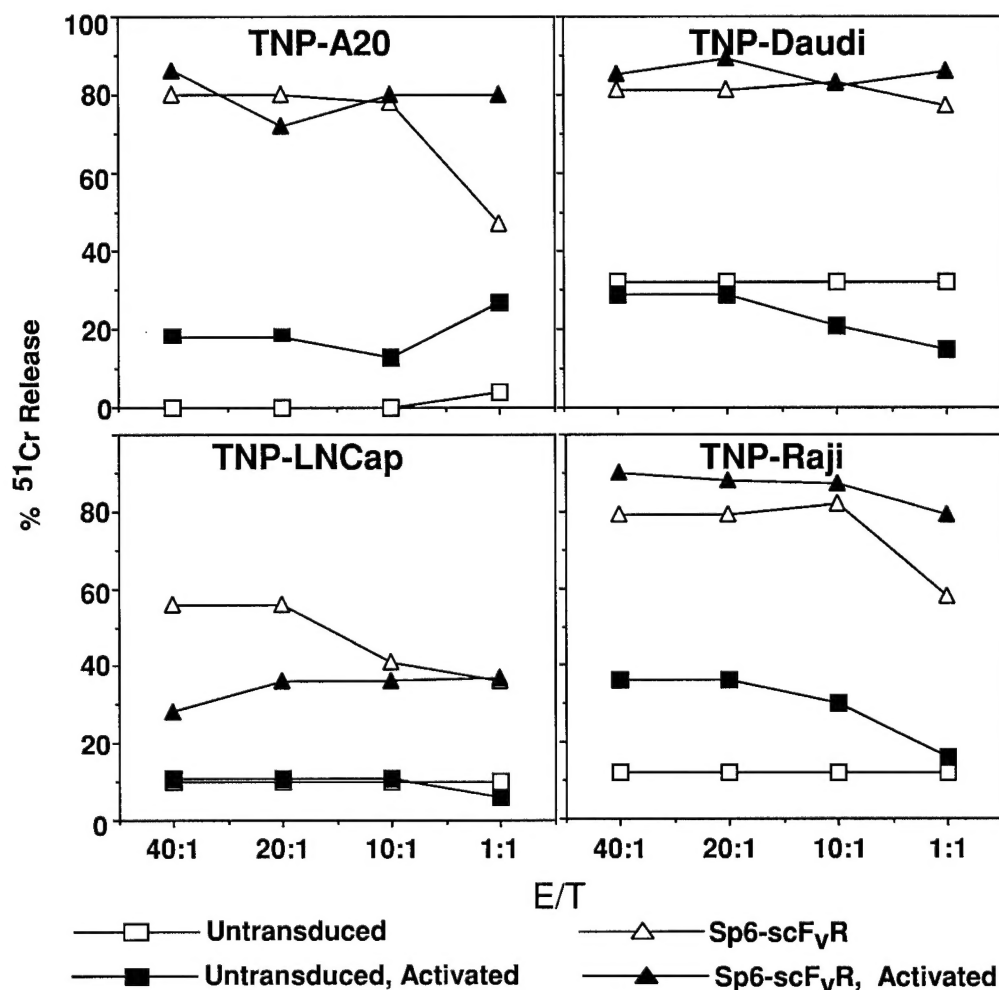
Fig. 6. Specific target cell cytotoxicity mediated by the chimeric receptor expressing lymphocytes. Lymphocytes were harvested 3 weeks after their transduction with chimeric receptor containing vectors. Part of the cells were reactivated on anti-CD3 and anti-CD28 antibodies 24 h before the assay. As target cells served various cell lines modified and unmodified by TNP. Specific ^{51}Cr release from the targets following incubation at different ratios with effector cells (E/T ratio) was determined following 7 h.

ty to kill specific target cells for at least 3 weeks in tissue culture, a period long enough to produce sufficient cells for patient treatment. These results set the grounds for in vivo testing of the T-body approach in animal models for cancer immunotherapy and later in patients.

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